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Mate Competition Drives Aggressive Behaviour in Female *Drosophila*

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To my heroes, Mom and Dad

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This thesis is a distillation of 7 years' worth of intensive labour and lots of ups and downs. It could not have come about without the contributions of a great many people with whom I am fortunate to have crossed paths.

I am most grateful to my parents, to whom this thesis is dedicated. Your irreplaceable and unconditional love and support mean everything to me. It certainly helped push through the lowest lows. Also, my sister for being the very best through all these years. I love you all.

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Título

Competição por Parceiros Sexuais Incita Comportamento Agressivo nas Fêmeas de *Drosophila*

Resumo

A agressão é um conjunto de comportamentos adaptativos que permitem aos animais competirem uns contra os outros num ambiente de recursos limitados. Em *Drosophila* tais comportamentos agressivos têm sido extensivamente estudados nos machos. Apesar de trabalho recente ter realçado a defesa territorial nas fêmeas, agressão em fêmeas de *Drosophila* é ainda pouco compreendida. De facto, se as fêmeas competem por parceiros sexuais, como os machos o fazem, tem permanecido uma incógnita. Na presente tese, reportamos que fêmeas de *Drosophila melanogaster* mostram consistemente comportamentos agressivos contra pares *in copulo*, contudo sem acarretar consequências de fertilidade nem fecundidade, quer na fêmea agressora, quer na fêmea alvo.

No segundo capítulo exploramos os estados internos e sinais externos que poderão regular a agressão das fêmeas, assim como neurónios sensoriais olfactivos candidatos que possam fazer a ligação entre um e outro. Mostramos que o comportamento agressivo de fêmeas está positivamente associado com a motivação reprodutiva da fêmea e que está fortemente dependente do sentido de olfacto. Para além disso, mostramos que o odor de comida, em combinação com o odor de conspecíficos mediado pelo receptor olfactivo OR47b são requisitos para a expressão adequada de comportamento agressivo em fêmeas.

Em suma, descrevemos um contexto social ligado à reprodução no qual fêmeas de *Drosophila* prontas a copular produzem consistentemente comportamentos agressivos de forma estereotipada. Estes resultados abrem o caminho para questões adicionais relevantes aos mecanismos neurais que governam este comportamento.

Abstract

Aggression is an adaptive set of behaviours that allows animals to compete against one another in an environment of limited resources. In *Drosophila* such aggressive behaviour has been extensively studied in males. Despite recent work highlighting territorial defence in females, female aggression in *Drosophila* is still poorly understood. Indeed, whether females compete for mating partners, as males do, has remained unknown so far. In this thesis, we report that *Drosophila melanogaster* females reliably display aggression towards mating pairs although without any bearing on either the aggressor's or the target's fertility or fecundity.

In the second chapter, we explore the internal states and external cues likely to regulate female aggression, as well as the olfactory sensory neuron candidates that might link one to the other. We show that female aggressive behaviour is positively associated with the female's mating drive and relies heavily on olfaction. Furthermore, we found that food odour in combination with OR47b-dependent fly odour sensing are required for proper expression of aggressive behaviour.

Taken together, we describe a social context linked to reproduction in which *Drosophila* females aspiring to mate produce consistent and stereotyped displays of aggression. These findings open the door for further inquiries into the neural mechanisms that govern this behaviour.

Author Contributions

Miguel Gaspar (MG) and Maria Luísa Vasconcelos (MLV) conceived and designed the project. MG performed all experiments and data analysis in this thesis. MG wrote this thesis.

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List of Abbreviations

5-HT: Serotonin

CHCs: Cuticular Hydrocarbons

cVA: 11-cis-Vaccenyl Acetate

dsx: doublesex

fru: fruitless

GR: Gustatory Receptor

IQR: InterQuartile Range

IR: Ionotropic Receptor

JH: Juvenile Hormone

OR: Olfactory Receptor

OSN: Olfactory Sensory Neuron

OSR: Operational Sex Ratio

PRR: Potential Reproductive Rate

RHP: Resource Holding Potential

SP: Sex Peptide

VMHvl: ventrolateral part of the VentroMedial Hypothalamus

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Chapter 1

General Introduction

Competition is a major selection force that shapes the evolution of phenotypic traits and behavioural strategies. In an ideal environment each individual would have at its disposal any amount of resources required to maximize its fitness. However, resource distribution in nature is not only limited, but also spatially and temporally heterogeneous. This unequal distribution means that those individuals that can gain access to and control these resources will gain a significant fitness advantage and, therefore, any traits that allow organisms to supplant competitors in securing said resources are highly adaptive. Much of what animals do to resolve competition is called aggression, which can be defined as an offensive physical action, or threat, to force others to abandon something they own or might achieve (Hickman et al., 2008). Aggressive behaviour is a common and highly recognizable behaviour exhibited by a variety of animal species (Breiehagen and Slagsvold, 1988; Clutton-Brock, 2007, 2009; Clutton-Brock and Huchard, 2013; Emlen et al., 1989; Emlen, 2008; Hohmann and Fruth, 2003; Hoogland, 1985; Huntingford and Turner, 1987; Kravitz and Huber, 2003; Pandolfi et al., 2021; Sandell, 1998; Stockley and Bro-Jørgensen, 2011) and is widely regarded as an important component of an animal's behavioural repertoire, having strong and persistent effects on various fitness-related traits (Figure 1.1). These include territory gain and defence, mate acquisition, parental care, intra- and inter-specific interactions, and anti-predation behaviour (While et al., 2009).

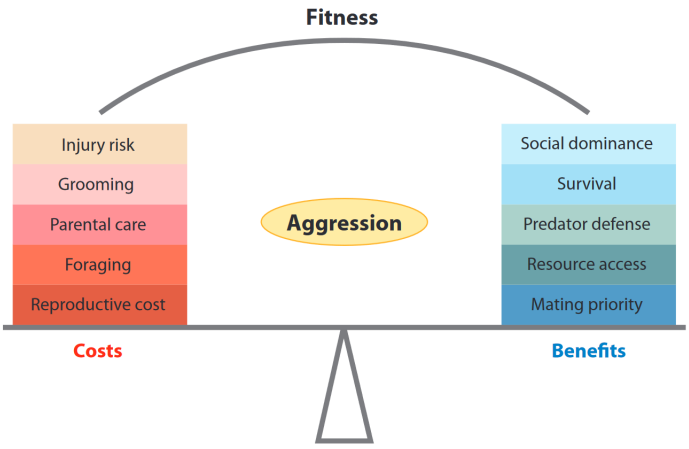


***Figure 1.1*: Schematic representation of the fitness costs and benefits of aggressive behaviour to individuals.**
The advantages of aggressive behaviour (right) include, at a more proximate level, defence and survival from predators, while ultimately allowing access to sparse resources, priority mating with high quality mates, and the establishing of dominance or the elevation of social rank. These benefits are offset by several fitness costs (left), such as the risk of injury or death, and the fact that time and energy spent on aggression cannot be redirected to other adaptive tasks, like grooming, foraging, or parental care, and may ultimately affect reproductive output. Taken from (Anholt and Mackay, 2012).

Together with feeding, fleeing, and reproduction, fighting is part of the so-called "four F's" of biology that are considered to govern most of animal behaviour, since these behaviours are critical for any animal's survival (Lorenz, 1966). These innate behaviours are, by definition, genetically predetermined, highly stereotyped and performed without prior experience or learning by all individuals of the species (Tinbergen, 1951). Despite the robustness imparted by this predetermination, innate behaviours are nonetheless flexible. By deploying different behavioural sub-programs, animals can select the appropriate set of actions to cope with ever-changing environmental, social, and internal contexts. Aggressive behaviour is no exception to this. Although aggression can yield competitive advantages, it is time-consuming and can be dangerous, and when exaggerated, persistent or expressed out of context, it can lead to serious wounding or death. To avoid such unnecessary injurious results, selection has favoured strategies that deescalate highly asymmetric contests. However, aggression cannot be altogether avoided, and in such instances, spectacular displays take place.

1.1 Types of Aggression

1.1.1 Inter-Specific Aggression

Inter-specific aggression occurs between members of different species. Such interactions can be generally grouped into three broad categories of predator-prey interactions (Lorenz, 1966):

Predator attacks – Perhaps the most common observable animal interaction in the wild will occur in those instances where a predator strikes at its prey in order to feed itself or its brood (Figures 1.2a and 1.2b). As we describe in the section below, those weapons that animals use to hunt, gather food, or protect themselves from predation are seldom the ones they will employ when engaged in fierce competition within their own kind.

Prey mobbing – In some instances, prey species will band together in groups and swarm would-be predators to chase them away or otherwise defend themselves (Figures 1.2c and 1.2d). Many birds will mob an owl if they find one in the daytime and drive it so far away that it will hunt somewhere else the next night. In some species this behaviour has the added effect of teaching naïve individuals in the group which animals are to be seen as dangerous and to be avoided.

Critical reactions – Animals will also fight heterospecifics if they find themselves cornered without a means for escape or when escape is otherwise prevented by strong social ties, such as those which forbid the animal to abandon its brood or family. In such cases that flight is prohibited, and freezing will no longer conceal its presence, an animal finds no alternative other than to fight back (Figures 1.2e and 1.2f).

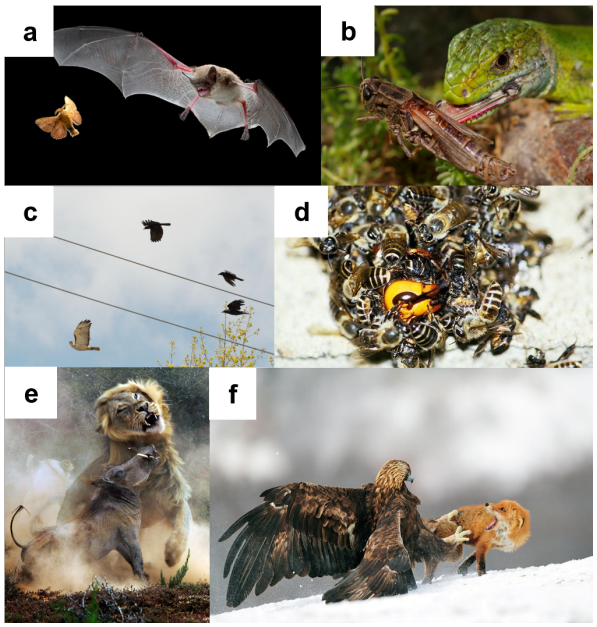


***Figure 1.2*: Natural occurrences of inter-specific aggression.**
(a) and **(b)**: examples of predators attacking and feeding on prey. **(a)** A bat about to capture a moth. **(b)** A western green lizard (*Lacerta bilineata*) feeding on a grasshopper. **(c)** and **(d)**: examples of prey mobbing against would-be predators. **(c)** American crows (*Corvus brachyrhynchus*) mobbing a red-tailed hawk (*Buteo jamaicensis*), chasing it away. **(d)** Japanese honey bees (*Apis cerana japonica*) mobbing an intruding giant hornet (*Vespa mandarinia*), overheating it to death. **(e)** and **(f)**: examples of critical reactions. **(e)** A warthog (*Phacochoerus africanus*) counterattacks a charging lion (*Panthera leo*) in an attempt to escape. **(f)** A red fox (*Vulpes vulpes*) reacts to a swooping golden eagle (*Aquila chrysaetos*) by adopting an aggressive posture. Image sources: **(a)**: `https://pixels.com/featured/little-brown-bat-hunting-moth-michael-durham.html`; **(b)**: `https://en.wikipedia.org/wiki/Predation`; **(c)**: `https://en.wikipedia.org/wiki/Mobbing_(animal_behavior)`; **(d)**: `https://www.quora.com/How-do-Japanese-bees-protect-themselves-from-Japanese-giant-hornets`; **(e)**: `https://www.earthtouchnews.com/natural-world/predator-vs-prey/photographer-captures-warthogs-epic-clash-with-a-lion/`; **(f)**: `https://pixels.com/featured/golden-eagle-and-red-fox-yves-adams.html`.

1.1.2 Intra-Specific Aggression

Darwin's "struggle for existence" is sometimes mistakenly interpreted as applying solely to the struggle between different species. However, organisms will invariably struggle more frequently and more intensely under the day-to-day competition found between those individuals that are most closely related, that is to say, those belonging to the same species. Since these will share

the most similarities in terms of resource requirements, resource acquisition methods, niche occupation, and behavioural habits, competition will be most severe among conspecifics (Darwin, 1859; Lorenz, 1966). Aggressive behaviour thus mediates intra-specific competition for food, reproductive opportunities, shelter, and territory (which can often provide all of the former). Among social animals, aggressive displays also serve as way to establish stable hierarchies in which the most dominant individuals have priority access to those resources (Anholt and Mackay, 2012; Bell et al., 2012; Buston, 2003; Clutton-Brock, 2007; Faulkes and Bennett, 2001; French and Inglett, 1989; Young et al., 2006).

1.1.3 Intra-Sexual Aggression

Within animal populations, competition is often particularly acute among individuals of the same sex because such individuals require the same limited resources to maximize their reproductive success. For example, adult females may require safe nest sites or other limited resources for reproduction (Clutton-Brock et al., 2006; Clutton-Brock, 2007; Dunbar and Sharman, 1983; Lewis et al., 2004; Pandolfi et al., 2021; Robinson and Kruuk, 2007; Rosvall, 2008; Sommer, 2010; Stockley and Campbell, 2013; Watson and Simmons, 2010; Yasukawa and Searcy, 1982), whereas adult males often compete for mating opportunities with a limited number of sexually receptive females (Chen et al., 2002; Clutton-Brock, 2007; Jung et al., 2020; Lewis et al., 2004; Pandolfi et al., 2021; Sommer, 2010). Competition between same-sex individuals, or intra-sexual competition, is therefore a widespread evolutionary selection pressure. This pattern emerges due to asymmetric potential reproductive rates (PRR), which, in turn, skew the operational sex ratio (OSR) of the population to be male-biased (Kvarnemo and Ahnesjö, 1996). In the majority of animals, while males only need to overcome a relatively short refractory period before becoming available to mate again, fertilized females will gestate the progeny for several weeks to several months, during which they are reproductively unavailable. Therefore, even with an initially balanced sex ratio, over time, the amount of reproductively active males will tend to remain approximately constant, while the amount of reproductively active females will dwindle with successive matings. Under these circumstances, males have a higher PRR than females, and therefore the OSR, the ratio of animals of each sex that can actively pursue reproduction, becomes male-biased. It is this imbalance that drives males to compete for sexual partners, while fe-

males will try to compensate gestation costs (and lactation costs in the case of mammals) by competing for food sources.

1.2 Sexual Dimorphisms of Aggressive Behaviour

1.2.1 Male-Male Aggression

Male-male competition (henceforth male competition) for access to females is a universal feature of animal behaviour (Andersson, 1994; Clutton-Brock, 2007; Darwin, 1871; Pandolfi et al., 2021). If aggression is so prevalent, how then do species ensure that they do not lead themselves to extinction through systematic injury or death when competing? To this common problem, evolution has repeatedly converged on a common solution – ritualized armaments displays. Males of many animal species will sport some sort of weapon they can wield against competitors when engaged in aggressive encounters (Emlen, 2008). These usually take the form of horns, antlers, pincers, or other often exaggerated morphological characteristics (Figure 1.3). However, these weapons are seldom intended to kill or otherwise irreversibly cripple competitors, but are many times, as the expression goes, "for show". Armed individuals very often engage in ritualized displays to communicate their fighting ability and in turn assess would-be competitors. Typically, body size, and by extension, weapon size, translates into increased fighting capacity, and therefore a higher aptitude to hold on to resources. This capacity is often referred to as resource holding potential (RHP). Thus, through ritualized displays, competing members of the same sex with asymmetric RHPs can avoid direct confrontation altogether, as low RHP individuals have little hope of overcoming the competition, and thereby avoid potentially serious injuries. Direct confrontation will thus emerge where RHPs are most similar, and therefore where the weapon's signal is most ambiguous. These are also the cases where, given their similar fighting capacity, individuals are less likely to be seriously injured. Weapons have thus a two-fold function. First, they function as a signal. Since armaments are metabolically expensive to develop and maintain (Emlen, 2008), weapon status is often an honest signal of fitness and high RHP. For competing males this serves as a potential deterrent, while at the same time, for females this can be an honest signal of male quality. Second, when aggression reaches its consummatory phase, weapons serve as a tool for resource acquisition or retention. For exam-

ple, male crickets use their stridulations to convey information to both males and females, signalling their fighting ability as well as their reproductive quality, respectively (Kravitz and Huber, 2003). That animals have the ability to select the appropriate course of action depending on the communicated signals of other conspecifics again speaks to the plasticity of aggressive behaviour.

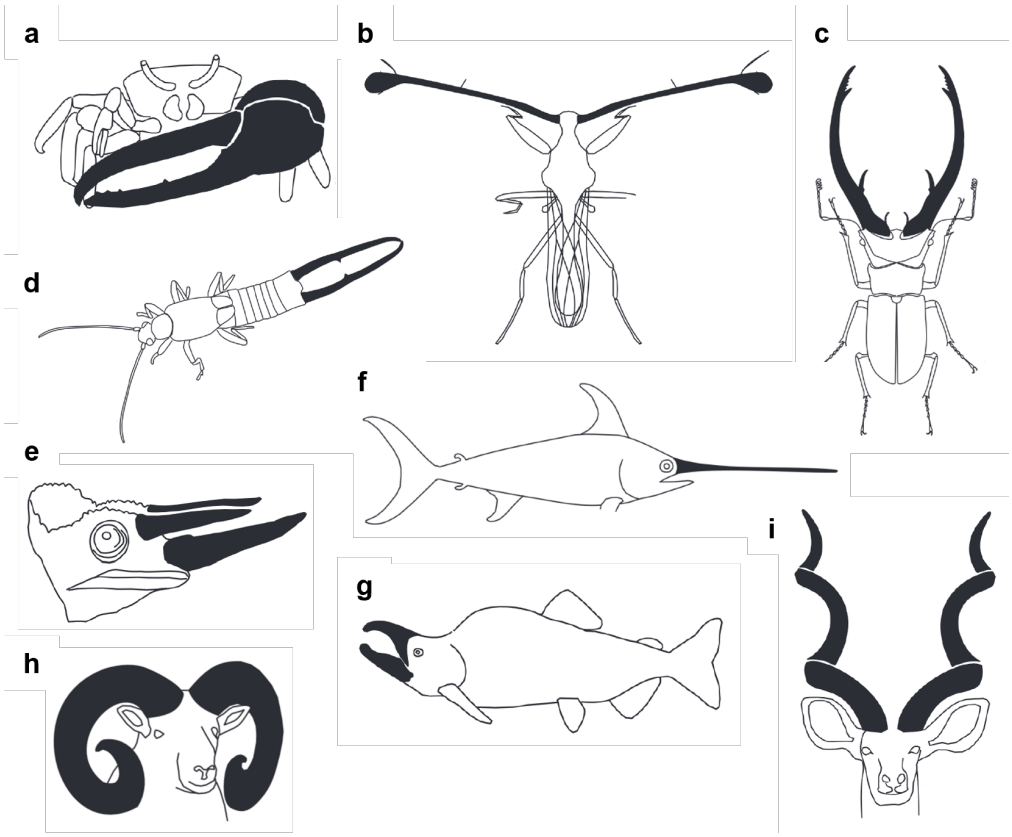


***Figure 1.3*: Male weaponry employed in intra-sexual competition is prevalent across the animal kingdom.**
Schematic representation of various animals and associated weaponry. Black-filled areas highlight the body parts that act as weapons. Weapon diversity is reflected both in the amount of animal taxa it can be found in, but also in its shape, size, and body part used. **(a)** Atlantic marsh fiddler crab (*Minuca pugnax*); **(b)** Malaysian stalk-eyed fly (*Teleopsis dalmanni*); **(c)** A stag beetle (*Cyclommatus* sp.); **(d)** An earwig (*Forficula* sp.); **(e)** Three-horned chameleon (*Chamaeleo jacksonii*); **(f)** Swordfish (*Xiphias gladius*); **(g)** Red salmon (*Oncorhynchus nerka*); **(h)** Bighorn sheep (*Ovis canadensis*); **(i)** Greater kudu (*Tragelaphus strepsiceros*). Adapted from (Emlen, 2008). Images not to scale.

1.2.2 Female-Female Aggression

To date, the majority of work on aggression has largely concentrated on the behavioural and physiological links between aggression and fitness-related traits in males. By contrast, understanding of intra-sexual competition among females has been slow to develop. Female aggression is assumed to be either subtle or inconspicuous and has received relatively little attention. Recent work, however, suggests that female aggression may have important consequences in a number of functional contexts, such as territory acquisition and eviction (Bell et al., 2012; Buston, 2003; Clutton-Brock et al., 2006; Rosvall, 2008; Young et al., 2006), the maintenance of monogamy (Dunn and Hannon, 1991; Sandell, 1998), and retention of parental care (Breiehagen and Slagsvold, 1988; Emlen et al., 1989; Petrie, 1983; While et al., 2009; Yasukawa and Searcy, 1982) (Figure 1.4). In females, because the same selection pressures that drive male-male aggression typically do not apply, their fights tend to be more subtle but result in more impactful outcomes. The lack of ornaments and weaponry with which to signal their fighting capacity means that females will more often escalate aggressive encounters, incurring more serious injury from these encounters. The outcome of female competition can come in many forms, including competitor displacement (Bebié and McElligott, 2006; Buston, 2003; Karvonen et al., 2000), copulation disruption (Bro-Jørgensen, 2002; Hohmann and Fruth, 2003; Sommer, 2010), reproductive suppression (Bell et al., 2012; Buston, 2003; Clutton-Brock et al., 2006; Faulkes and Bennett, 2001; French and Inglett, 1989; Stockley and Bro-Jørgensen, 2011; Wasser and Barash, 1983; Young et al., 2006), kidnapping (Silk, 1980), and infanticide (Emlen et al., 1989; Hoogland, 1985; Müller and Eggert, 1990; Pusey and Schroepfer-Walker, 2013).

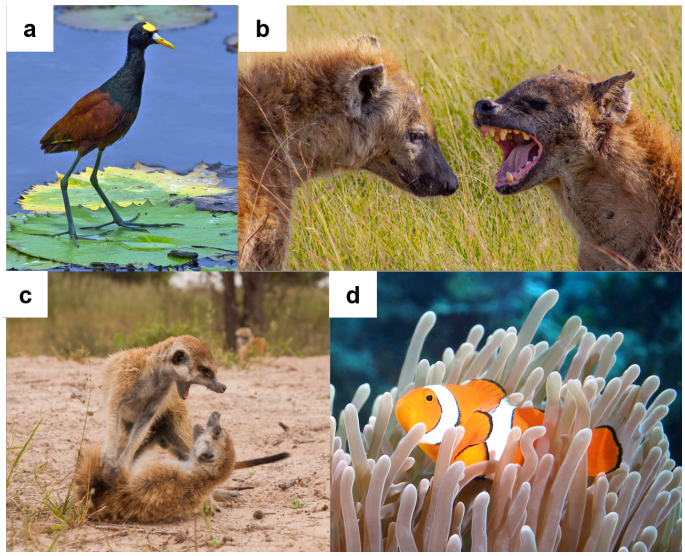


***Figure 1.4*: Natural examples of female aggression across species.** Female-female aggression is present in animals from a multitude of different taxa. **(a)** In the northern jacana (*Jacana jacana*), females hold territories that attract males, which invest heavily in parental care. In order to defend their male harems, females patrol their territories, evicting invading female competitors. Successful invaders will also often kill the broods of previous females. **(b)** Females of the spotted hyena (*Crocuta crocuta*) fight for social rank, which affects mating and feeding order. **(c)** Kalahari meerkat (*Suricata suricatta*) females fight to maintain their status as the dominant female of the colony, ensuring their role as the sole breeding female. **(d)** Similarly, in the orange clownfish (*Amphiprion percula*), the dominant female will evict competing females, thus monopolizing reproduction. Image sources: **(a)**: `https://pixels.com/featured/northern-jacana-larry-linton.html`; **(b)**: `https://pixels.com/featured/hyena-breath-test-michael-howard.html`; **(c)**: `https://phys.org/news/2018-08-breeder-meerkats-age-faster-subordinates.html`; **(d)**: `https://www.biodiversity4all.org/observations/52135391`.

The high energetic demands of gestation mean that the reproductive success of females is often constrained by the availability of resources and females thus often compete directly for food, threatening or attacking other individuals that feed close to them, or for access to feeding territories (Clutton-Brock and Huchard, 2013; Hoogland, 1985; Stockley and Campbell, 2013). Female-female aggression (henceforth female aggression) over reproduction appears then to occur where the resources necessary for successful reproduction, are limited, specifically 1) the food or breeding territories necessary for successful pregnancy and weaning, 2) infant care from mates or helpers, and 3) good quality mates or sperm (Breiehagen and Slagsvold, 1988; Cheney et al., 2012; Clutton-Brock and Huchard, 2013; Dunn and Hannon, 1991; Lewis et al., 2004; Petrie, 1983; Robin-

son and Kruuk, 2007; Sandell, 1998; Shelly, 1999; Watson and Simmons, 2010; While et al., 2009; Yasukawa and Searcy, 1982). As would be expected, the frequency of overt female competition for mating partners increases in populations where adult sex ratios are strongly biased towards females, where there is a high degree of reproductive synchrony, or where females mate with multiple partners (Bath et al., 2021; Cheney et al., 2012; Stockley and Bro-Jørgensen, 2011). In some polygynous ungulates where males initially compete intensely for access to females, oestrous females can subsequently compete aggressively for the attentions of favoured males (e.g., topi, *Damaliscus lunatus* (Bro-Jørgensen, 2002; Stockley and Bro-Jørgensen, 2011)). Similarly, in langurs (*Presbytis entellus*), females can interfere as frequently as males to disrupt copulations (Sommer, 2010), whereas dominant female meerkats (*Suricata suricatta*) induce stress-based reproductive suppression in subordinate females to retain them as helpers, while also actively evicting potential rivals (Young et al., 2006). In spite of these accounts in the wild, our understanding of the factors involved in the regulation of female aggressive behaviour remains poor. By leveraging an organism with stereotyped and genetically tractable behaviours, we can systematically study aggressive behaviour in more detail.

1.3 Aggression in *Drosophila*

Drosophila melanogaster presents a powerful model for the dissection of complex traits, including aggression, because: 1) large numbers of individuals of the same genotype can be reared rapidly under controlled environmental conditions; 2) it boasts a complex, yet genetically tractable, nervous system; and 3) its aggressive actions are stereotypically fixed, making behavioural quantifications much more amenable. Thus, the study of *Drosophila* affords opportunities to investigate how nervous systems of higher complexity introduce flexibility into a rich behavioural repertoire (Kim et al., 2017).

1.3.1 Aggressive Behaviour

The behavioural contexts of male competition over territory and mates, as well as female competition over food and egg-laying sites have been used extensively in the studies of aggression in the fruit fly. Males and females are known to display a diverging, though somewhat overlapping, set of aggressive actions

(Nilsen et al., 2004). Males fight employing lunging, boxing, and tussling as the highest-intensity aggressive actions, and establishing clear hierarchical dominance relationships (Kim et al., 2018; Nilsen et al., 2004; Vrontou et al., 2006). Indeed, winning males are prone to continue winning subsequent fights, while the opposite is true of the losers (Kim et al., 2018; Simon and Heberlein, 2020; Vrontou et al., 2006). This loser effect, also reported in other animals (Egge and Swallow, 2011), is so strong that a single defeat completely abolishes the hyper-aggressive phenotype of males specifically bred for hyper-aggression (Penn et al., 2010). In contrast, females fight using headbutting and shoving, as the highest-intensity aggressive actions (Nilsen et al., 2004), and do not establish dominance hierarchies (Vrontou et al., 2006). The cues that promote aggression also differ between the sexes (Figure 1.5). The presence of a decapitated female in the food patch will increase fighting intensity in males (Chen et al., 2002), whereas females will fight more vigorously in the presence of live yeast (Ueda and Kidokoro, 2002). In males, the perception of male-specific pheromones (such as 11-cis-vaccenyl acetate (cVA) and 7-tricosene), as well as the sound of conspecifics have been shown to regulate aggression (Liu et al., 2011; Versteven et al., 2017; Wang and Anderson, 2010; Wang et al., 2011). Analogous signals in females have not yet been identified. Another interesting feature of female aggressive behaviour is that it is strongly stimulated by mating (Bath et al., 2017, 2020), which is consistent with the notion that gravid females fight for resources that improve the odds of their offspring.

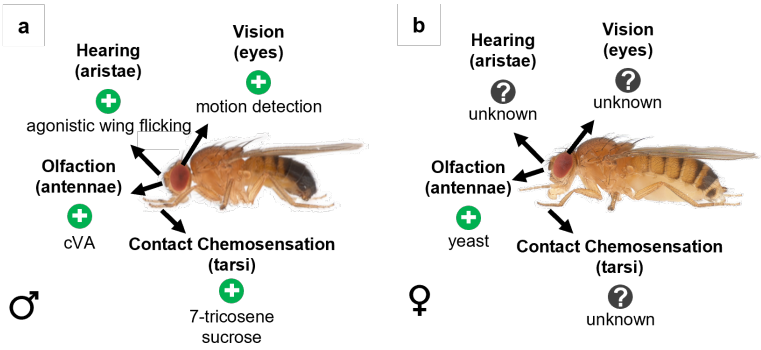


***Figure 1.5*: Sensory modalities involved in *Drosophila* aggression.**
(a) and **(b)**: Sensory modalities and organs involved in the detection of aggression-promoting ((⊕)) or aggression-inhibiting (⊖) cues in males and females, respectively.

1.3.2 Aggressive Circuitry

Importantly, since the fly is such a powerful genetic model system, the study of fly aggressive behaviour has been accompanied by an impressive body of work dissecting the neuronal circuits underlying aggression. The sex determination genes *fruitless* (*fru*) and *doublesex* (*dsx*) are instrumental in ensuring the proper establishment of sexually dimorphic circuitry in the adult brain (Figure 1.6). They have thus been extensively studied, having been shown to govern mating behaviour in *Drosophila* (Auer and Benton, 2016; Billeter et al., 2006; Demir and Dickson, 2005; Fabre et al., 2012; Grosjean et al., 2011; Ishii et al., 2020; Jois et al., 2018; Kallman et al., 2015; Kimura et al., 2008; Koganezawa et al., 2016; Kohatsu et al., 2011; Rezával et al., 2012; Rideout et al., 2010; Sethi et al., 2019; Stockinger et al., 2005; von Philipsborn et al., 2011; Zhou et al., 2014). More recent evidence has come to light implicating these genes in the regulation of aggressive behaviour as well (Anderson, 2016; Asahina et al., 2014; Certel et al., 2007; Chan and Kravitz, 2007; Deutsch et al., 2020; Ishii et al., 2020; Koganezawa et al., 2016; Palavicino-Maggio et al., 2019; Sengupta et al., 2022; Vrontou et al., 2006; Yuan et al., 2014). Male- and female-specific splicing of these genes is sufficient to confer sex-specific behaviours to flies (Auer and Benton, 2016; Chan and Kravitz, 2007; Datta et al., 2008; Demir and Dickson, 2005; Häsemeyer et al., 2009; Ishii et al., 2020; Rezával et al., 2012; Rideout et al., 2010; Stockinger et al., 2005; Vrontou et al., 2006; Wohl et al., 2020; Yang et al., 2009). In males, the Dsx+, Fru+ brain cluster, called P1, is a known regulator of courtship behaviour – activation of a cellular subset within this neural population induces courtship towards both females and other males indiscriminately (Anderson, 2016; Auer and Benton, 2016; Ishii et al., 2020; Kallman et al., 2015; Kimura et al., 2008; Kohatsu et al., 2011; Jung et al., 2020; von Philipsborn et al., 2011; Zhang et al., 2019). In addition to this, activation of these neurons is also responsible for driving aggressive behaviour in males (Anderson, 2016; Hoopfer et al., 2015; Ishii et al., 2020; Jung et al., 2020; Koganezawa et al., 2016). Interestingly, recent work has revealed that the behaviour elicited by these neurons is dose-dependent – weak activation induces aggression only, while strong activation leads to courtship behaviour -, suggesting a possible node for context-dependent modulation of social behaviours (Anderson, 2016; Hoopfer et al., 2015). Females lack P1 neurons, as these express the male-specific *fru* splice variant, absent in females. They do, however, like

males, possess a Dsx+, Fru- cluster, called pC1. Activation of specific cellular subsets of this brain region, in particular those labelled as pC1d, induces female aggressive behaviour and threat displays (Chiu et al., 2021; Deutsch et al., 2020; Koganezawa et al., 2016; Palavicino-Maggio et al., 2019; Schretter et al., 2020). Furthermore, serotonin, octopamine, and dopamine are among the biogenic amines affecting aggression in male *Drosophila* (Alekseyenko et al., 2013, 2014, 2019; Andrews et al., 2014; Certel et al., 2007; Dierick and Greenspan, 2007; Hoyer et al., 2008; Jia et al., 2021; Johnson et al., 2009; Yuan et al., 2014; Zhou et al., 2008), although the identity of such aminergic neurons and their relation to P1 and pC1 clusters remain unclear. Among the *Drosophila* neuropeptides, tachykinin and neuropeptide F have also been shown to promote male aggression (Asahina et al., 2014; Dierick and Greenspan, 2007; Wohl et al., 2020). Despite the strides made in the understanding of *Drosophila* circuits governing aggressive behaviour, the context of its study has so far remained focused on territorial defense. Indeed, for all the plasticity of female aggression other species have demonstrated, the contextual flexibility of *Drosophila* aggressive behaviour still begs exploring.

In this thesis I address competition between sexually receptive females for a mating partner under a female-biased sex ratio in the fruit fly *Drosophila melanogaster*. In the first part, I characterize female aggression under mate competition conditions as well as the putative fitness advantages of this behaviour under such circumstances. In the second part I investigate potentially relevant internal states and external cues that may contribute to aggression modulation. Finally, we target potential candidate olfactory sensory neurons (OSNs) as interfaces between the external and internal medium of the animal.

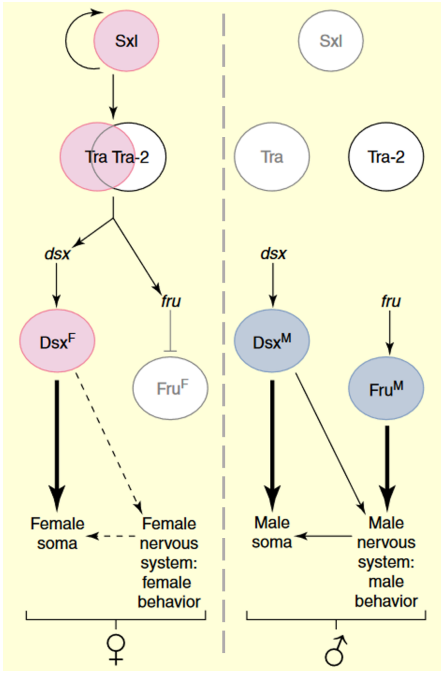


Figure 1.6: *fruitless* and *doublesex* specification cascade in male and female *Drosophila*.
Black lines or colors indicate active; grey indicates inactive or non-functional. Female-specific proteins are pink, male-specific proteins are blue, and non-sex-specific proteins are white. In females (left), the presence of two X chromosomes sustains auto-regulation of the *sex lethal* gene. Sex Lethal (Sxl) protein controls the female-specific splicing of the *transformer* gene to generate the Transformer (Tra) protein, which, together with Transformer-2 (Tra-2), regulates the female-specific splicing of *doublesex* (*dsx*) and *fruitless* (*fru*). Thus, a functional female-specific form of Doublesex (Dsx^F) is produced, along with a female-specific, non-functional form of Fruitless (Fru^F). In males (right), the absence of Tra results in *dsx* and *fru* being both spliced into functional male-specific forms, the Dsx^M and Fru^M proteins. The Fru protein is mostly required to wire sex-specific circuits in the nervous system that control sex-specific behaviours. Dsx protein is largely involved in determining sex-specific somatic structures and external morphology, and is also required for establishing sex-specific circuit in the nervous system responsible for the expression of some sex-specific behaviours. Adapted from (Billeter et al., 2006).

Chapter 2

Wild Type Female Aggression Towards Mating Pairs

2.1 Characterization of Aggressive Behaviour

To investigate whether females compete for males, we started by pairing naïve males with two virgin females and recorded their interactions for one hour. This allows us to capture both the entire courtship period leading up to copulation, as well as the entirety of copulation from start to finish. We define an aggression bout as any period of time where females display consecutive headbutts and/or shoving, the highest intensity, and therefore most visually discernible, aggressive behaviours in females (Nilsen et al., 2004; Ueda and Kidokoro, 2002; Vrontou et al., 2006) (Supplementary Video 4.3.1), and aggression rate as the percentage of copulation time that unmated females spend performing aggression. Encounters refer to moments when the unmated female is within 4mm of the mating pair, and aggressive encounters are those where aggression occurs. While we did not observe any kind of agonistic interaction between the animals while courtship is ongoing, we reliably found that their encounters (Supplementary Figure 4.1.1a) resulted in female aggressive behaviour during copulation, i. e., aggression towards the mating female and male (hereafter referred to as the mating pair) displayed by the unmated female. (Figures 2.1a, 2.1b, 2.1c, and Supplementary Figure 4.1.1b). We then decided to characterize when and how female flies display this behaviour. We started by investigating whether aggressive behaviour is displayed at random during copulation. If this were so, we would expect to not find any clear pattern of aggression distributed over copulation time. We found, however, that aggression is mostly concentrated in the first half of copulation (Figure 2.1d), around 5 to 9 minutes (Supplementary Figure 4.1.1c). Given that ejaculation is expected to occur within that period of time (Tayler et al., 2012), these findings are in line with a possible strategy to dismount the copulating male; alternatively, female aggression drive could decay drastically after the initial aggressive bouts. Once copulation ends, a new phase of courtship with the second female follows, which can culminate in copulation with that second female. However, the first mated female never displayed any aggression towards any of the other flies, either during courtship of the second female, or during the second copulation (data not shown). Next, we wondered whether aggressive females showed any preference in targeting either the mating male or the mating female. When identifying the target of each aggressive bout, we found that they are more or less evenly distributed between both sexes (Figure 2.1e). It thus seems that despite the high

levels of aggressive displays and its focus on early copulation, females target both mating individuals indiscriminately. Next, we used positional information gathered from tracking data to further characterize female behaviour during aggressive bouts. The angle between flies is defined as the angle formed at the intersection of the lines that represent the orientation of each of the two females (Figure 2.2a, bottom). We found that females perform aggressive displays with angles between the flies comprised in a narrow range, mostly from 60° to 120° (Figure 2.2a, top). These results reveal that rather than head-to-head attacks, typically observed in male-male aggression, females attack the flanks of mating pairs. As expected, the facing angle of the aggressive female, i. e., the angle formed between the line that represents the aggressive female's orientation and the line that unites both females' centroids (Supplementary Figure 4.1.1d, bottom) lies mostly between 0° and 30° (Supplementary Figure 4.1.1d, top), confirming that this is an oriented behaviour. Moreover, we found that when displaying aggression towards the mating pair (Figure 2.1e), aggressive females preferentially target the posterior half of the mating female (Figure 2.2b), which is where the mating male is also located. Whether this specific targeting is an adaptive strategy employed by the female to affect the reproductive outcome of the ongoing copulation, remains unclear. Taken together, we have shown that females reliably fight during copulation of a mating pair in a stereotypical fashion, flanking the posterior half of the mating female. We next investigated whether these aggressive displays have any bearing in the reproductive output of either female.

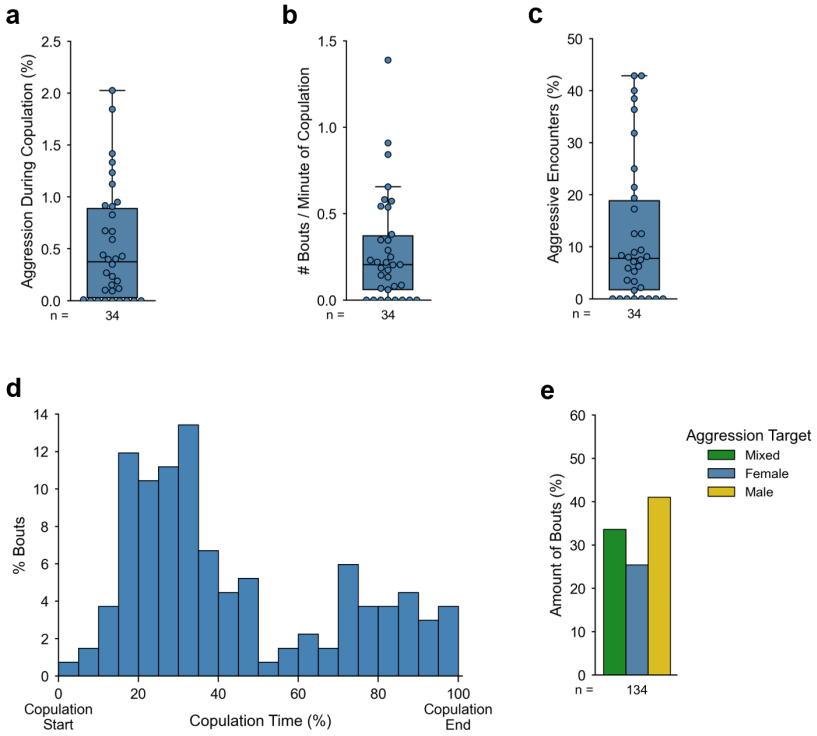


***Figure 2.1*: Female aggressive behaviour is reliable and stereotyped in the presence of a mating pair.**
(a) Aggression rate of wild type females towards a mating pair. **(b)** Number of aggression bouts per minute of copulation displayed by wild type females towards a mating pair. **(c)** Percentage of encounters where aggression occurs. Encounters are defined as moments when the non-mating female is within 4mm of the mating pair. **(d)** Distribution of aggression occurrence during copulation. Copulation time is expressed as a percentage to normalize for different copulation durations. 0% represents the start of copulation and 100% the end of copulation, irrespective of copulation duration. n = 134 aggression bouts from 34 tested females. **(e)** Percentage of aggression bouts targeting the mating female, the mating male, or both. n = 134 aggression bouts from 34 tested females.

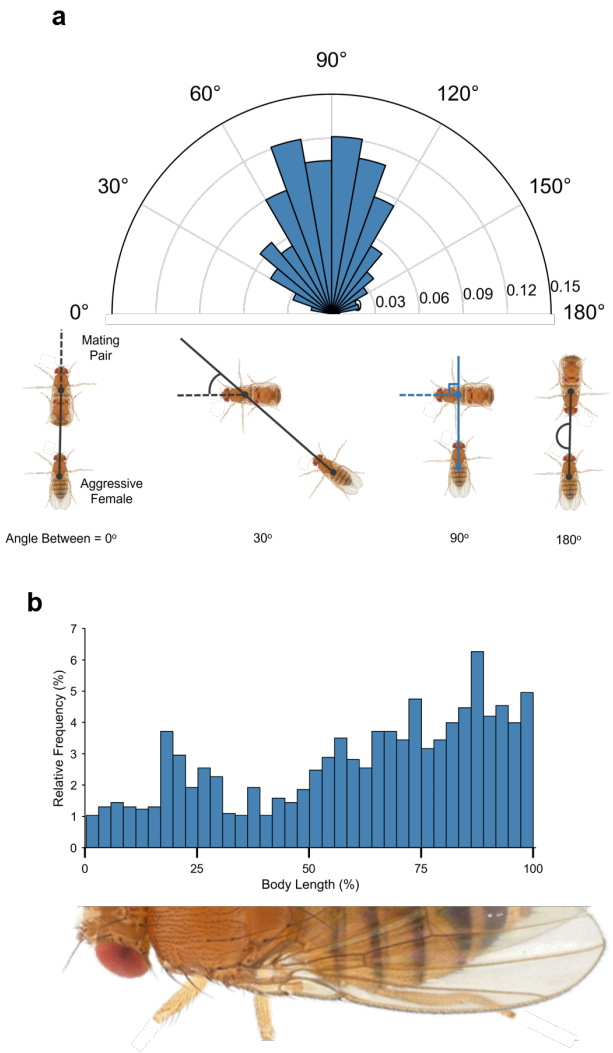


***Figure 2.2*: Female aggressive behaviour is reliable and stereotyped in the presence of a mating pair *(continued)*.**
(a) Distribution of angles between flies. Top: the polar axis represents the range of possible angles, while the radial axis represents the percentage of total angles that fall within any given range. Angles are binned in 10-degree intervals. Bottom: schematic of representative facing angles. Full lines represent the orientation of the aggressive female; dashed lines represent the orientation of the target mating pair. Angle between flies is the angle made by the intersection of those two lines. Blue lines match the range of angles found experimentally. **(b)** Distribution of points along the mating female's body axis that are targeted during aggression. The body axis is represented as the percentage of fly length to normalize for variation in female body size. Sample size (number of flies, unless otherwise stated) is shown under each graph, as well as in Supplementary Table 4.4.3.

2.2 Biological Explanations for Female Aggressive Behaviour

From our previous findings (Figures 2.1d and 2.2b), we asked whether the observed aggressive displays could have some effect on the reproductive outcomes of either the target or aggressive female. To address this question, we compared three different contexts: mating pairs together with an aggressive female, such that normal aggressive interactions can occur ("with aggression", Supplementary Video 4.3.2); mating pairs separated from a competing female by a mesh, such that they occupy different halves of the same arena, thereby preventing aggressive interactions ("partition", Supplementary Video 4.3.3), the mesh being removed after copulation ends to allow the competitor female to also mate; or mating pairs without any additional competitors, such that there is no aggression and mating pairs are not disturbed by any other potential signals coming from the competing female ("single couple"; see Experimental Procedures). If the purpose of aggressive displays is to cause the male to dismount sooner from the current mating, possibly curtailing ejaculation, we would expect to see a reduction in the duration of the first copulation. This is not the case, as we found that copulation duration remains undisturbed in the presence or absence of aggression (Figure 2.3a). Alternatively, if aggressive displays are a strategy to prime the male so that the second copulation can start sooner, then we would expect to see a decrease in the latency to the second mating. However, we found that the latency to the second copulation is unaffected by the presence or absence of female aggression (Figure 2.3b). So far, we looked at potential short-term consequences of aggression. However, we reasoned that aggressive displays might have more long-term implications, specifically at the level of the number of eggs laid and progeny fitness. Aggressive behaviour could be used as a mechanism to lower the fitness of the competitor; additionally, such aggressive displays could also incur a high metabolic cost and therefore directly impact the reproductive capacity of the aggressive. To explore this, we collected both females (target and aggressive) after each experiment and counted the number of eggs laid by each one after 24 hours. In addition, we also counted the number of total adults that eclosed from those laid eggs and used that to calculate the hatching rate (see Experimental Procedures). Surprisingly, we found no difference either in the number of eggs laid, or their hatching rate of the target female in the presence or absence of aggression (Figures 2.3c and

2.3d), suggesting that being subjected to aggressive displays has no bearing on egg production or viability. Similarly, the number of eggs laid and their hatching rate of the aggressive female were also unaffected in the presence or absence of aggression (Figures 2.3e and 2.3f), implying that even if aggression is energetically taxing, it is not to the point of limiting egg production or viability. The possibility also exists that the amount of aggressive behaviour displayed over an hour-long experiment is simply insufficient compared to the natural life history of a fly in the wild to produce measurable effects. Although we could not uncover the biological consequences of this aggressive behaviour, the consistency with which we can observe it and the stereotyped nature of its execution hint at its biological relevance.

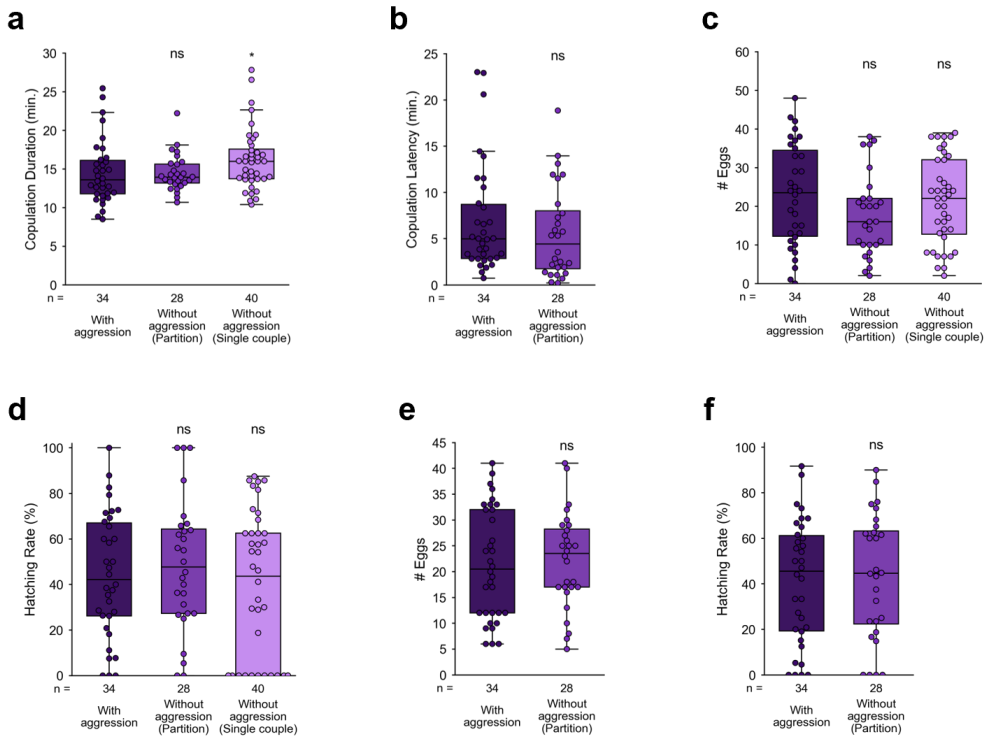


***Figure 2.3*: Aggression does not affect copulation duration, latency to the second copulation, number of eggs laid, or hatching rate.**
(a) Duration of the first copulation (target female). **(b)** Latency to the second copulation (competitor female). **(c)** Number of eggs laid from the first copulation (target female). **(d)** Hatching rate from the first copulation (target female). **(e)** Number of eggs laid from the second copulation (competitor female). **(f)** Hatching rate from the second copulation (competitor female). All statistical output was provided by Mann-Whitney U tests, or Student's t tests for independent groups, where applicable. To account for multiple comparisons effects, Bonferroni correction was applied post-hoc whenever two or more groups were compared; ns = not significant, *$p < 0.05$. Sample size (number of flies tested) is shown for each condition below its corresponding boxplot. See Supplementary Table 4.4.3 for exact p-values, effect sizes, and statistical tests and assumptions used.

Chapter 3

Contextual Modulation of Female Aggressive Behaviour

3.1 The Internal State

The fact that virgin females are aggressive during the first copulation, but then no aggression is displayed by the recently mated female towards the second copulation lead us to hypothesize that female aggressive drive might be modulated by the female's mating status, one of the component of an animal's internal landscape. The behavioural changes associated with the transfer of sex peptide (SP) from the male sperm during mating are well documented, leading, within 24 hours, to drastic shifts in egg laying, receptivity, feeding preference, and even fighting for food patches, for instance (Bath et al., 2017; Chen et al., 1988; Häsemeyer et al., 2009; Hussain et al., 2016; Liu and Kubli, 2003; Peng et al., 2005; Ribeiro and Dickson, 2010; Walker et al., 2015; Yang et al., 2009; Yapici et al., 2008). It is therefore reasonable that the same, or similar mechanisms might be at play here, modulating aggressive behaviour between the virgin and mated states of the female. Additionally, it has been recently reported that the experience of copulation itself is enough to induce an early onset of behavioural changes, although through an independent mechanism than that of the SP pathway (Shao et al., 2019). To test whether the mating status-related changes also affect female aggressive displays, we quantified the aggression rate of 24h-mated females, as well as 2h-mated females. We found that in both cases female aggression levels were strongly reduced (Figure 3.1a, Supplementary Figure 4.1.2a, and Supplementary Figure 4.1.2b). This effect is not simply due to a general lack of activity of mated females, since these animals walk as much as virgin controls (Figure 3.1b), suggesting that not only the SP-mediated changes in female physiology, but also short-term mating experience are sufficient to significantly impact aggressive drive. Is the post-mating reduction in female receptivity, and presumably in the mating drive, the leading cause for lower aggression? To test whether a low mating drive is associated with low levels of female aggression, we decided to study the effect of courtship deprivation on female aggressive drive, an alternative manipulation of mating drive. We quantified the aggression rate of virgin females that were not courted by a male (i.e., courtship deprived) and that were only introduced in the behavioural arena during the ongoing copulation of a mating pair. We observed that depriving the females of exposure to courtship is also enough to significantly reduce their aggressive displays (Figure 3.1a, Supplementary Figure 4.1.2a, and Supplementary Figure 4.1.2b). These

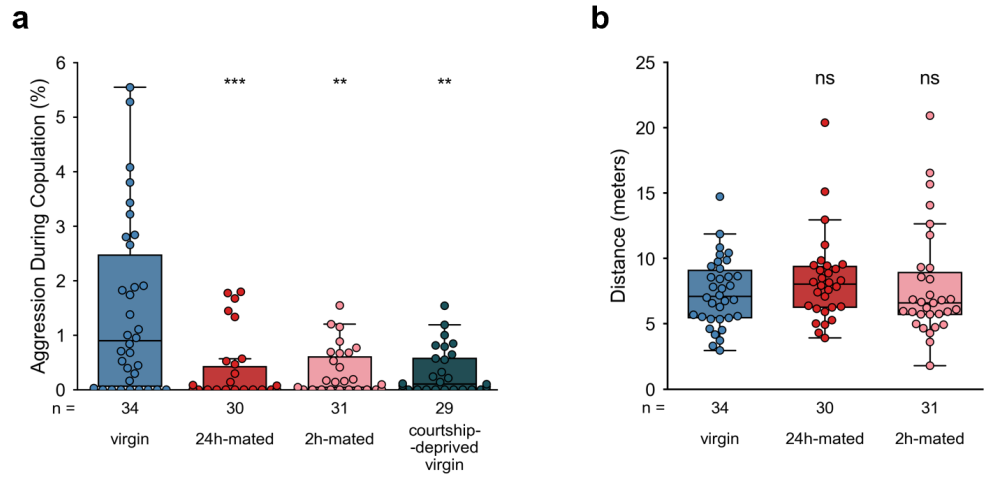


***Figure 3.1*: Mating drive regulates aggressive behaviour.**
(a) Aggression rate of wild type virgin, 24h-mated, 2h-mated, and courtship-deprived virgin females towards a mating pair. **(b)** Distance walked by non-mating females, from the start of the experiment until the end of copulation. Sample size (number of flies tested) is shown for each condition below its corresponding boxplot. All statistical output was provided by Mann-Whitney U tests, or Student's t tests for independent groups, where applicable. To account for multiple comparisons effects, Bonferroni correction was applied post-hoc whenever two or more groups were compared; ns = not significant, $^{**}p < 0.01$, $^{***}p < 0.001$. Sample size (number of flies tested) is shown for each condition below its corresponding boxplot. See Supplementary Table 4.4.3 for exact p-values, effect sizes, and statistical tests and assumptions used.

results show that mating drive and aggression drive are positively associated in females.

3.2 The External Environment

Having pinpointed one component of the internal state of the female fly that regulates its aggressive behaviour, we next sought to investigate which elements in the external environment of the fly could influence this behaviour. To address this question, we tested the contribution of taste, hearing, vision, and olfaction with the use of either mutant or otherwise manipulated flies (see Experimental Procedures). We found that visual mutants and, more strikingly, olfactory mutants show a strong decrease in aggressive behaviour compared to wild type females, stressing the importance of these two sensory modalities (Figure 3.2a, Supplementary Figure 4.1.3a, and Supplementary Figure 4.1.3b). We have shown before that aggressive displays are a targeted behaviour, i.e., fe-

males orient themselves towards their intended target (Figure 2.2d). However, if that is the only role played by vision in aggression, then we would expect to see females displaying aggression when they are not in proximity of the mating pair. We found that blind females display aggression exclusively towards the mating pair, suggesting that vision is likely gating aggression in some other way, perhaps allowing females to visually recognize a mating pair of flies. The effect of removing hearing did not reach statistical significance, but we do note that it is nonetheless quite large, reducing aggression by around 75% (Figure 3.2a, Supplementary Figure 4.1.3a, and Supplementary Figure 4.1.3b; see Supplementary Table 4.4.3). This is in line with previous reports of hearing regulating aggressive behaviour in fruit flies (Versteven et al., 2017). One possible explanation for this effect is that acoustic stimulation by courtship song increases the female mating drive, and by removing hearing, and therefore keeping mating drive low, aggression drive remains accordingly low. Alternatively, given recent work showing that females sing during copulation (Kerwin et al., 2020), aggressive displays might be partially driven by the detection of the mating female's song. Only the removal of taste clearly shows no effect on female aggression (Figure 3.2a, Supplementary Figure 4.1.3a, and Supplementary Figure 4.1.3b). We also found that the aggression effects observed in each of the sensory conditions are not a reflection of the flies' general lack of activity, since no group of flies walks significantly less than wild type control flies (Figure 3.2b). Therefore, olfaction seems to be the primary sensory modality that females require in order to identify the appropriate environment in which to perform aggressive displays.

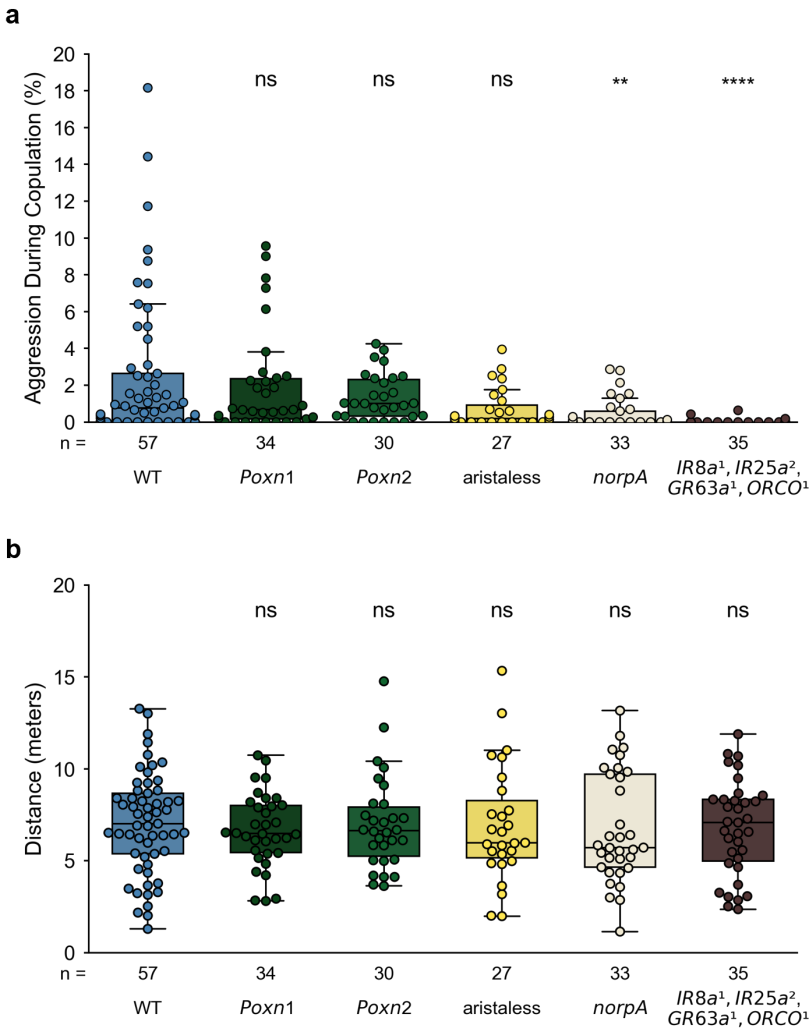


***Figure 3.2*: Olfaction is required for normal levels of aggression.**
(a) Aggression rate of wild type (WT), tasteless (*Poxn1* and *Poxn2*), deaf (aristaless), blind (*norpA*), and anosmic ($IR8a^1$, $IR25a^2$, $GR63a^1$, $ORCO^1$) virgin females towards a mating pair. **(b)** Distance walked by non-mating females, from the start of the experiment until the end of copulation. All statistical output was provided by Mann-Whitney U tests, or Student's t tests for independent groups, where applicable. To account for multiple comparisons effects, Bonferroni correction was applied post-hoc whenever two or more groups were compared; ns = not significant, **p < 0.01, ****p < 0.0001. Sample size (number of flies tested) is shown for each condition below its corresponding boxplot. See Supplementary Table 4.4.2 for detailed fly genotypes, and Supplementary Table 4.4.3 for exact p-values, effect sizes, and statistical tests and assumptions used.

3.3 The Olfactory System: Interface between External and Internal Contexts

We next sought to narrow down the number of possible candidates within the olfactory landscape of the female fly that may modulate aggressive behaviour. Customarily we lace all arenas with food paste prior to experiments, which is then removed, leaving only an odour trace of the food in the arenas (see Experimental Procedures). Food odour has been shown to stimulate male courtship (Grosjean et al., 2011), which is a critical component of our experiments, given that they depend on successful copulation. Since males are stimulated by the smell of food and given that both males and females fight for territories (Bath et al., 2017; Lim et al., 2014; Nilsen et al., 2004; Ueda and Kidokoro, 2002; Versteven et al., 2017), we reasoned that food odour could be acting as a representation of a territory, and could therefore be able to regulate female aggressive behaviour. To test this hypothesis, we compared the aggression rate of females in the presence of a mating pair, either with or without food odour. We found that food odour is indeed required for aggressive behaviour to be displayed (Figure 3.3a and Supplementary Figure 4.1.4a). This could mean that food odour does indeed act as a proxy for the presence of a territory and that the behaviour we have characterized thus far is in fact a fight for territory. If this were the case, then removing the male, and therefore copulation, should still yield high levels of aggressive behaviour in the presence of food odour only. We found that in the absence of a mating pair, exposure to food odour by itself is not enough to induce the high levels of aggression observed in the presence of both components (compare Figure 3.3b and Supplementary Figure 4.1.4b with Figure 3.3a and Supplementary Figure 4.1.4a). This suggests that food odour together with the presence of a mating pair are both needed for high number of aggressive displays to take place, which implies that some additional olfactory cue from the mating pair is needed in addition to that of food odour.

Olfactory and ionotropic receptors have extensively been shown to regulate many fly social behaviours, including reproductive and aggressive behaviours (Billeter and Wolfner, 2018; Dweck et al., 2015; Grosjean et al., 2011; Hussain et al., 2016; Kohlmeier et al., 2021; Kurtovic et al., 2007; Lin et al., 2016; Liu et al., 2011; Lone and Sharma, 2012; Lone et al., 2015; Sethi et al., 2019; van der Goes van Naters and Carlson, 2007; Wang et al., 2011; Zhuang et al., 2016; Ziegler et al., 2013). However, the fly olfactory system is comprised of dozens of

such receptors. Hence, we needed an approach to narrow down the number of potential candidates. It is well established that the *Drosophila* gene *fruitless* encodes sexually dimorphic behaviour, including aggression (Asahina et al., 2014; Certel et al., 2007; Chan and Kravitz, 2007; Ishii et al., 2020; Koganezawa et al., 2016; Vrontou et al., 2006; Wohl et al., 2020), by specifying sex-specific circuits during development (Billeter et al., 2006; Datta et al., 2008; Demir and Dickson, 2005; Ishii et al., 2020; Kimura et al., 2008; Stockinger et al., 2005; Vrontou et al., 2006). Three *Drosophila* olfactory receptor neurons express the *fruitless* transcript (Stockinger et al., 2005; Zhang et al., 2020): IR84a, which senses a specific range of chemicals present in the odour blend of food, and shown to be responsible for the food-stimulating effect on male courtship (Grosjean et al., 2011); OR67d, which detects the male-specific pheromone 11-cis-vaccenyl acetate (cVA), shown to be a strong mediator of male-male aggression (Kurtovic et al., 2007; Wang and Anderson, 2010); and OR47b, which senses compounds present in both males and females, and is a known modulator of receptivity in females and courtship in males (Dweck et al., 2015; Kohlmeier et al., 2021; Lin et al., 2016; Lone et al., 2015; Lone and Sharma, 2012; Sethi et al., 2019; van der Goes van Naters and Carlson, 2007; Zhuang et al., 2016; Ziegler et al., 2013). Silencing IR84a-housing OSNs had no effect on female aggression (Figure 3.3c and Supplementary Figure 4.1.4c). Given that IR84a-expressing OSNs are tuned to a very narrow range of food-related odours, these findings indicate that other olfactory receptors are responsible for the food odour-derived modulation of aggression. Silencing OR67d-expressing neurons likewise did not affect aggression rate in a significant way (Figure 3.3d and Supplementary Figure 4.1.4d), suggesting that, surprisingly, the male-specific olfactory cue cVA seems to not be necessary for the proper display of female aggressive behaviour. Only the silencing of OR47b-expressing OSNs resulted in a significant decrease, of around 40%, in female aggression rate (Figure 3.3e, Supplementary Figure 4.1.4e, and Supplementary Table 4.4.3). We conclude from these results that multiple olfactory cues are required for the proper expression of female aggressive behaviour. These include olfactory cues likely include those from food, via as-yet uncharacterized receptors, and those from other flies, through the activity of OR47b-expressing olfactory neurons.

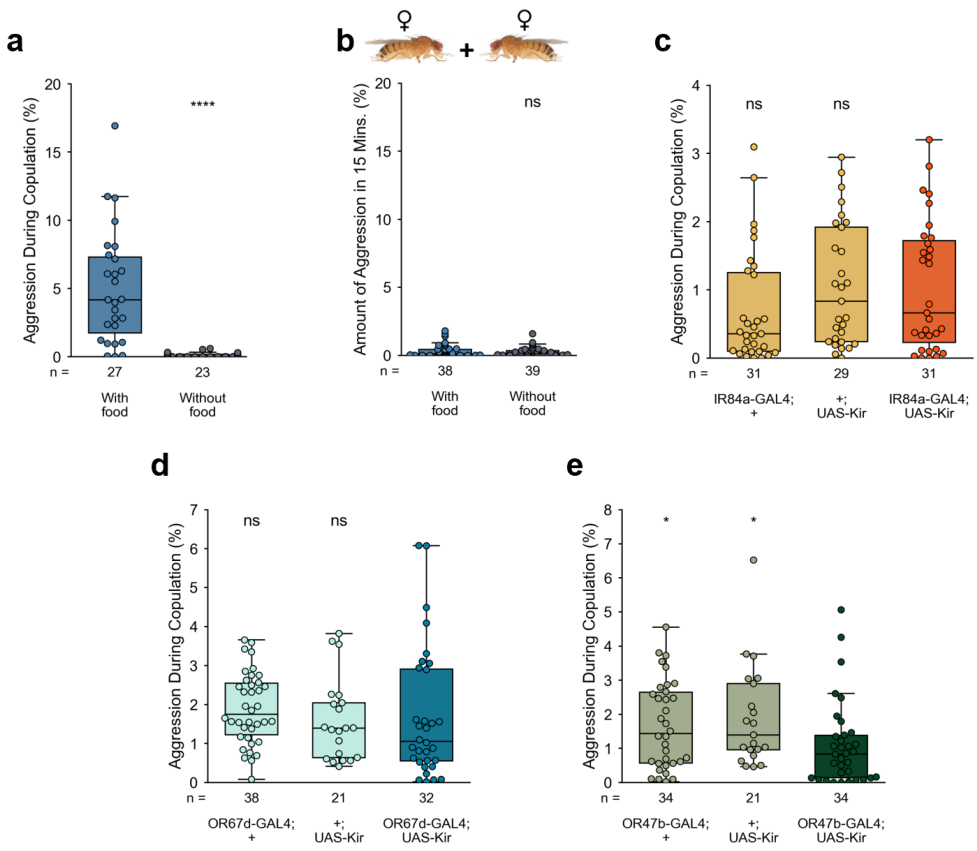


***Figure 3.3*: Presence of food odour and activity of OR47b olfactory sensory neurons contribute to female aggressive behaviour.**
Aggression rate in the following conditions: **(a)** Wild type flies in the absence or presence of food odour. **(b)** Wild type female pairs, without a male, in the absence or presence of food odour. **(c)** Flies with silenced IR84a-expressing OSNs and respective controls in the presence of food odour. **(d)** Flies with silenced OR67d-expressing OSNs and respective controls in the presence of food odour. **(e)** Flies with silenced OR47b-expressing OSNs and respective controls in the presence of food odour. All statistical output was provided by Mann-Whitney U tests, or Student's t tests for independent groups, where applicable. To account for multiple comparisons effects, Bonferroni correction was applied post-hoc whenever two or more groups were compared; ns = not significant, *$p < 0.05$, ****$p < 0.0001$. Sample size (number of flies tested) is shown for each condition below its corresponding boxplot. See Supplementary Table 4.4.2 for detailed fly genotypes, and Supplementary Table 4.4.3 for exact p-values, effect sizes, and statistical tests and assumptions used.

Chapter 4

General Discussion

Aggressive behaviour occurring in the context of intra-sexual competition is an important trait for animal fitness as it allows animals to compete for limited resources. In this thesis, we have shown that females of *Drosophila melanogaster* will compete for mates, specifically by engaging in aggressive displays towards mating pairs. These displays are stereotyped and strongly dependent on olfactory cues from both food and other flies, with a significant, yet partial, contribution of OR47b-expressing olfactory sensory neurons.

4.1 Internal State and Resource Valuation

Previous work in female *Drosophila* aggression has focused on competition over territory, where two females are fighting over a physical patch of food. Interestingly, in that context mated females are more aggressive than virgin females (Bath et al., 2017, 2020, 2021), in stark opposition with our own findings in the context of mate competition. This reversal in the relationship between mating status and aggression levels could be due to a shift in the females' perceived value of available resources: virgin females would value mating partners higher than egg-laying sites, whereas mated females would prioritize egg-laying site acquisition over possible early rematings. Indeed, it has been well established that the mating status of female *Drosophila* is capable of reversing the valence of resources, with mated females displaying a strong dietary yeast and salt preference in contrast to the significant sugar preference of virgin counterparts (Ribeiro and Dickson, 2010; Walker et al., 2015). It is therefore reasonable that a similar mechanism might regulate female aggression levels as a function of mating status and available resources.

Our finding that food odour, in addition to the presence of a mating pair, is required to drive aggression in virgin females suggests that this behaviour is contingent on an ecologically relevant context, as either cue by itself fails to elicit appropriate aggressive responses. Whether this is because, as reported in males (Grosjean et al., 2011), food odour has a stimulating effect that increases female mating drive, or the odour itself is representative of a nearby egg-laying site remains to be elucidated. Using carefully defined food recipes where key factors are omitted (e.g. yeast extracts) or, conversely, composed of a single class of ingredients (e.g. sugar only) can help identify which olfactory components enhance female aggressive behaviour in this context, and whether mating drive and egg-laying are enhanced by the same or disparate components.

4.2 Sensing the Environment and Modulating Behaviour

Our study reveals a strong dependence of aggressive behaviour on olfaction, largely contributed to by OR47b, which is tuned to a select few elements of flies' cuticular hydrocarbon profiles. A growing body of work has been uncovering the many roles this olfactory receptor plays in mediating *Drosophila* reproductive interactions, from young female preference and mating advantage in males, to increased mate choosiness in females (Kohlmeier et al., 2021; Lin et al., 2016; Lone et al., 2015; Zhuang et al., 2016). In fact, the behavioural differences we observed between virgin and mated females fit very well with the described physiological modulation of OR47b OSNs occurring after mating. Briefly, juvenile hormone (JH) production induced by mating alters the sensitivity of OR47b neurons, changing the behavioural output to the same sensory input depending on mating status of the individual (Kohlmeier et al., 2021). Just as desensitized OR47b neurons impart increased choosiness in mated females, this mechanism could just as easily gate aggressive behaviour by dampening aggression-triggering olfactory stimuli in mated females; virgin females, on the other hand, would integrate the information provided by more sensitive OR47b neurons with their internal mating drive in order to express an appropriate aggressive response. Artificial manipulation of JH receptors in OR47b OSNs of virgin and mated females under mate competition could provide direct evidence for a common JH-mediated mechanism of behavioural regulation.

While using anosmic females lead to a complete abrogation of aggressive behaviour, impeding the function of OR47b-expressing OSNs produced only a partial reduction in female aggression. It is clear that additional olfactory sensors are involved in evaluating the correct sensory context in which to execute aggressive behaviour. Given our findings that food odour is essential for driving aggression, these additional receptors are most likely ones tuned to relevant food odour components. Besides olfaction, vision, and likely hearing, were also revealed to have an impact in female aggression levels. This is in line with previous work reporting the involvement of these modalities in male aggression (Duistermars et al., 2018; Hoyer et al., 2008; Versteven et al., 2017), suggesting even more parallels with female aggression. Males require a moving object to express aggression (Duistermars et al., 2018), whereas in females vision is likely used to distinguish the general shape of mating rivals, since we did not observe

any instance of aggression targeted anywhere but towards the mating pair. As for acoustic signals, it has been reported that males respond with aggressive displays to agonistic sounds of rival males (Versteven et al., 2017). In the context of female mate competition, aggressive females might be responding to the copulation song generated by the mating female (Kerwin et al., 2020). Thus, females might be using acoustic, visual, and olfactory cues as long-, mid-, and close-range signals to identify potential competitors and execute appropriate aggressive levels towards them. Whether such a system is actually being employed by females, and which specific features of the environment each modality is picking up on to contribute to female aggressive displays is still unclear. Manipulating some of the sensory components in the environment, such as presenting perfumed dummies or mute copulating females might reveal the identity and contribution of the relevant environmental features regulating female aggression.

4.3 Possible Upstream Circuitry for Mate-Driven Aggression

In order to accumulate sensory information and internal states (mating, feeding, thirst, etc.) over time, the nervous system requires some form of integrator circuit onto which these external and internal signals can converge in order to dictate appropriate behavioural action. In *Drosophila*, pC1 is a known key integrator node that regulates reproductive and aggressive behaviours in *Drosophila*, with different cell types contributing to different behaviours (Chiu et al., 2021; Deutsch et al., 2020; Ishii et al., 2020; Koganezawa et al., 2016; Schretter et al., 2020; Wang et al., 2020, 2021; Zhou et al., 2014). pC1d in particular has been shown to be responsible for driving aggressive displays in females, with other cell types mediating functions related to receptivity (Chiu et al., 2021; Deutsch et al., 2020; Schretter et al., 2020). In addition to this, recurrent activity of pC1 has been shown to generate a persistent internal state in the fly brain, thus providing a neural basis for context-specific stimulus integration (Jung et al., 2020; Zhang et al., 2019; Zhou et al., 2014). It is therefore likely that this same circuitry is being recruited in the context of our study, regulating mate-driven aggressive competition and possibly weighing the contribution of olfactory, auditory, and visual sensory inputs together with the internal state of the female.

In a striking parallel to the described aggression circuitry in *Drosophila* females, recent work in mice has shown that different neural subpopulations within the ventrolateral area of the ventromedial hypothalamus (VMHvl) modulate female sexual and aggressive behaviours in a mating state-dependent manner (Lee et al., 2014; Lin et al., 2011; Liu et al., 2022). Moreover, olfactory circuits have been reported to have an essential function in mouse aggressive behaviour, by specifically regulating approach to conspecific volatiles (Hashikawa et al., 2016). Given the seeming evolutionary convergence of aggression-regulating circuits in the central brain of mice and flies, it sounds reasonable that our olfactory mutant results reflect a possible conservation of the sensory pathways that feed into those central circuits.

4.4 Ecological Considerations

Many fruit flies exhibit lekking mating systems, including *Tephritidae* and Hawaiian *Drosophila* species (Papadopoulos et al., 2009; Shelly, 2018). These mating systems are characterized by four main features: 1) males provide no parental care, and supply only gametes; 2) males are spatially aggregated in mating areas, or leks; 3) males do not control access to resources critical to females; and 4) females are free to select mates at the lek (Shelly, 2018). Although the current knowledge of *Drosophila melanogaster* ecology remains scant, and field evidence is lacking regarding whether it is a true lekking species, it does exhibit some of its elements. Despite being uncommon, this type of mating system is taxonomically widespread, being present in other insects, crustaceans, fish, reptiles, birds, and mammals (Bro-Jørgensen, 2002; Clutton-Brock et al., 1988; Croll and McClintock, 2000; Karvonen et al., 2000; Mindy Nelson, 1995; Oakes, 1992; Papadopoulos et al., 2009; Shelly, 2018; Soto and Trites, 2011; Turner, 2015; Vitousek et al., 2007). In these systems, males will perform courtship displays to females, who visit the leks for the sole purpose of mating. Under these circumstances inter-female competition, many times in the form of aggression, takes place. However, paralleling our own findings, the biological significance of aggressive behaviours in some of these species is still poorly understood. One reason for aggression may be to reduce the waiting time for access to a preferred male, where the cost of aggression would be lower than delaying mating with a high fitness partner or mating with a lower fitness partner. Alternatively, aggression could be used as a way to reduce the fitness of rival females by, for

example, disturbing copulation. Finally, aggressive females might compete for high quality or quantity of sperm in a limited environment, therefore attempting to decrease sperm volume transferred to competitors. In our work we report no effect of female aggression on copulation duration, nor on the reproductive output of either female involved. It therefore seems that none of the strategies offered above are at play here. Indeed, active copulation disruption induced by female aggression seems rare across species (Bro-Jørgensen, 2002; Karvonen et al., 2000; Papadopoulos et al., 2009; Sommer, 2010). Even in the case of the Mediterranean fruit fly, *Ceratitis capitata*, where virgin females display intense aggression towards mating pairs, the advantage of executing such displays remains elusive (Papadopoulos et al., 2009). These may include negatively affecting sperm allocation to competitors in order to increase sperm volume or quality they might receive from harassed males in the future. Since agonistic interactions can be costly, they could also be functioning as a signal of female quality, with males preferring subsequent matings with females that display more vigorous aggressive behaviour.

In conclusion, we report in this thesis how female aggression can be elicited by mating pairs in the presence of food odour and highlight the importance of social context in the characterization of behaviour. These findings pave the way for addressing the neural underpinnings of female aggressive behaviour in a social context linked to reproduction and add to the growing body of evidence that *Drosophila* females display rich, complex behaviours that are sensitive to social, environmental, and internal states.

Chapter 5

Experimental Procedures

5.1 Resource Availability

5.1.1 Materials availability

This study did not generate new unique reagents. The materials built in-house are open-source and can be made available through our institute's scientific hardware platform (Champalimaud Hardware Platform; `http://www.cf-hw.org/`). Non-electrical components used in building the setup are listed in Supplementary Table 4.4.5 and a schematic of the fully built setup can be found in Supplementary Diagram 4.2.1.

5.1.2 Data and code availability

Raw movies supporting the current study have not been deposited in a public repository because of their large size, but annotation files in csv format generated from acquired videos as well as necessary Python code used to generate all data present in the current thesis have been deposited on Harvard Dataverse and are publicly available (`https://doi.org/10.7910/DVN/URA528`). Original, raw movies, as well as any additional information required to reanalyse the data reported in this paper are available on request from the Thesis Supervisor. *Drosophila melanogaster* images used throughout this work were taken from Nicolas Gompel's lab webpage (`http://gompel.org/images-2/drosophilidae`) under a Creative Commons license.

5.2 Fly Stocks and Husbandry

Fruit flies of the species *Drosophila melanogaster* were raised in standard cornmeal-agar medium, using Vienna food recipe (in 1L of water: 80g molasses-barley malt, 22g beet syrup, 80g corn flour, 18g granulated yeast, 10g soy flour, 8g agar-agar, 8mL propionic acid, 12mL 15% nipagin, 35mL Bavistin), at 25ºC and 70% relative humidity in a 12h dark:12h light cycle. Detailed information on fly stocks used and fly genotypes for each experiment are present in Supplementary Table 4.4.1 and Supplementary Table 4.4.2, respectively.

5.3 Behavioural Assays

For all experiments both male and female flies were collected under CO_2 anaesthesia and raised in isolation at 25°C and 70% relative humidity and aged 4-8 days until the day of the experiment. All experiments were performed at 25°C, 70% relative humidity, in dim light, and between Zeitgeber times 0 and 4. For experiments in Figures 2.1, 2.2, 3.1, 3.2, Supplementary Figures 4.1.1, 4.1.2, and 4.1.3, all flies were collected as late-stage pupae. For practical purposes, all flies were collected as early adults (1-3 hours post-eclosion) for all other experiments. All flies were inspected both at collection time and briefly before the start of experiments for any noticeable physical defects. Any fly that exhibited any of the following traits were discarded from being used in experiments: broken or bent tarsi, malformed legs, damaged or curled wings, wings locked at non-resting positions, bloated abdomens, abnormal walking patterns, markedly reduced walking, and black ommatidia anywhere in any of the eyes. To distinguish between the two females, we selected one of the females in each pair at random and painted them over the posterior half of the thorax and the scutellum with a metallic silver 0.8mm nib roller-ball pen (Uni-Ball Signo UM-120NM). To hold the females in place during painting, they were pinned by one of their midlegs using precision forceps (Fine Science Tools Dumont #5CO, item n°11295-20), applying as little force as necessary to avoid damage to the legs. To limit the effect of this procedure as a possible confounding factor, we also subjected males and unpainted females to CO_2 anaesthesia and manipulated them with the same precision forceps. After the painting procedure, all flies were allowed to recover at 25°C and 70% relative humidity for at least 36 hours before experiments. Unless stated otherwise, all experimental arenas were laced with fly food paste at least overnight to imbue the arenas with the smell of food to stimulate courtship. To prepare this paste, we added 1mL of milliQ water to a regular fly food vial of standard cornmeal-agar medium and physically mashed the food and water together with a small, 5mm-wide metal spatula until a consistent paste was formed. A small amount of this paste was transferred to each conical arena, or enough was transferred to fill the smaller, rectangular arenas. This paste was removed with the aid of paper towels as thoroughly as possible prior to aspirating flies into the arenas to start the acquisition of experiment movies.

5.3.1 Figures 2.1, 2.2, and Supplementary Figure 4.1.1

Experimental data in Figures 2.1, 2.2, and Supplementary Figure 4.1.1 originate from the same experimental group and dataset as that of Figures 3.1 and Supplementary Figure 4.1.2.

5.3.2 Figure 2.3

To ensure a lack of any aggressive behaviour towards the mating pair, we introduced a partition in the arenas to physically separate the mating pair from the non-mating female (see **Behavioural arenas** section below). Briefly, the experiments start with partition in place, and one male with one female are gently aspirated into one of the partition sides, while the second female is aspirated into the other side of the partition. After 30 minutes, which ensures mating has occurred, the partition is removed to allow the isolated female to mate. At the end of the experiment, each of the two females in each arena was gently aspirated and transferred to a vial of standard cornmeal-agar medium (using Vienna food recipe) and kept at 25°C and 70% relative humidity. After 24 hours, if the females were still alive, they were discarded, the number of eggs laid in the vial was counted, and the vials were returned to 25°C for another 9 days. At that point we counted the number of eclosed adults, keeping the vials at 25°C and counting newly eclosed adults every day for 4 to 6 days additional days. To ensure that both females were mated during the experimental period, movies were acquired for 1 hour.

5.3.3 Figure 3.1 and Supplementary Figure 4.1.2

To generate 24h-mated females we aspirated a naïve male into each female's vial 24 hours before the start of next day's experiments. To ensure that flies were mated, the females' vials were kept and incubated at 25°C and 70% relative humidity for up to 15 days and checked for eclosed progeny. To generate 2h-mated females, naïve males were added to the females' vials at the start of experiments, and left undisturbed for 2 hours, after which they were included in the last batch of experiments of the day. Mating occurrence was checked visually. For the added virgin condition, for each experiment we started by gently introducing a single virgin female and a single naïve male to the arena, then checked visually for the start of copulation, upon which we then gently

aspirated a second virgin female into the same arena where the initial pair just started mating. Movies were acquired for 1 hour.

5.3.4 Figure 3.2 and Supplementary Figure 4.1.3

To remove gustation, we crossed two different homozygous PoxNeuro genetic deletions to each other in both directions (*PoxN1* and *PoxN2*); to remove vision we used a homozygous mutant for the *norpA* gene; to remove olfaction, we employed a quadruple mutant for both of the ionotropic receptor co-receptors, IR8a and IR25a, the olfactory receptor co-receptor, OR83c, and the CO_2 receptor GR63a (see Supplementary Table 4.4.2 for detailed fly genotypes); finally, to remove hearing, we removed both aristae in female flies. To do this, individual flies were anesthetized with CO_2 approximately 24 hours before the experiment. Aristae were cut bilaterally at their base with micro scissors (World Precision Instruments) under a scope. Flies were allowed to recover at 25°C and 70% relative humidity until the experiment. Movies were acquired for 45 minutes.

5.3.5 Figure 3.3 and Supplementary Figure 4.1.4

For experiments in Figures 3.3a, 3.3b, as well as Supplementary Figures 4.1.4a and 4.1.4b, the "food" conditions are performed as all previous experiments, i. e., by lacing arenas with food paste prior to testing flies. For the "no food" conditions, arenas are not laced with the food paste. In 3.3b and Supplementary Figure 4.1.4b, since there is no copulation from which to take the first 5 minutes of for analyses, we instead annotated aggression during the first 15 minutes of the experiment. Movies were acquired for 55 minutes. For experiments in Figures 3.3c, 3.3d, 3.3e, as well as Supplementary Figures 4.1.4c, 4.1.4d, and 4.1.4e, IR84-, OR67d-, and OR47b-expressing olfactory sensory neurons were silenced using the inwardly rectifying potassium channel Kir2.1 that hyperpolarizes the neurons, thus preventing action potential formation (Baines et al., 2001). Movies were acquired for 30 minutes.

5.3.6 Behavioural arenas

Experiments in Figures 2.1, 2.2, 3.1, 3.2, as well as Supplementary Figures 4.1.1, 4.1.2, and 4.1.3 were recorded in a 2×2 array of custom-made circular arenas with a conical-shaped bottom, as previously described (Aranha et al.,

2017; Simon and Dickinson, 2010). These arenas are made of mechanically bored white polyoxymethylene, with 11° sloped walls, 4mm maximum height, and approximately 4cm of walking diameter, topped with clear acrylic lids. Refer to Supplementary Diagram 4.2.2 for additional details. Experiments in Figures 2.3, 3.3 and Supplementary Figure 4.1.4 were recorded in a 4×4 array of 20×17mm rectangular, clear acrylic arenas with approximately 17×10mm of walking area and 3mm height, topped with clear acrylic lids. Refer to Supplementary Diagram 4.2.3 for further details. For experiments in Figure 2.3, we constructed additional rectangular arenas with the same specifications as before but leaving a 0.6mm-wide slit on both sides of the arenas. Through these openings a nylon plastic mesh (SEFAR-NITEX® 06-500/38) was placed, separating one of the females from the other female and male. This partition can then easily be removed at any time during movie acquisition without interrupting the experiments.

5.4 Quantification and Statistical Analysis

5.4.1 Movie acquisition

Experiments were recorded in grayscale at 60 frames per second with a camera (Point Grey Flea®3 FL3-U3-32S2M) equipped with a 5mm MegaPixel fixed focal length lens (EdmundOptics®, stock n°64-867) mounted above the arenas. Movies were acquired in dim light using 940nm LEDs integrated in a custom electronic LED array board (LED array v3.0) with associated control board (LED array interface v1.0) and control software (HARP version v0.3) designed and built in-house (Scientific Hardware Platform) and a UV/VIS cutoff M43.0×0.75 machine vision filter (EdmundOptics®, stock n°89-839). A list of camera parts used can be found in Supplementary Table 4.4.5; a list of the camera settings used for recording fly behaviour during experiments can be found in Supplementary Table 4.4.6. Bonsai (Lopes et al., 2015) version 2.4.0 was used to acquire the videos. The workflow used in the acquisition can be found in Supplementary Diagram 4.2.4. Experiments in Figures 2.1, 2.2, 3.1, 3.2, as well as Supplementary Figures 4.1.1, 4.1.2, and 4.1.3 were recorded with a resolution of 960×940 pixels. Experiments in Figures 2.3, 3.3, and Supplementary Figure 4.1.4 were recorded with a resolution of 1248×1010 pixels.

5.4.2 Data processing

After videos related to Figures 2.1, 2.2, 3.1, 3.2, as well as Supplementary Figures 4.1.1, 4.1.2, and 4.1.3 were acquired, FlyTracker (Eyjolfsdottir et al., 2014) was used to track the three flies and output information concerning their position, orientation, velocity, distance to the other fly, facing angle, and angle between flies. Because FlyTracker only provides angular and distance information for two animals, we adapted its code to ensure that these features were made available pairwise for each pair in our three fly assays (adapted code available at Harvard Dataverse: `https://doi.org/10.7910/DVN/URA528`). Subsequently, the in-house developed PythonVideoAnnotator (`https://biodata.pt/python_video_annotator`) was used to manually annotate the time and duration of copulation and aggression bouts. Aggression bouts were annotated as any moment where continuous headbutt or shoving (the highest intensity, and therefore most visually discernible, aggressive displays in females (Nilsen et al., 2004)) were observed, in-between which wing flicking, and intense fencing would occasionally also be included, if they occurred. Bouts were considered separate instances when the flies would stop interacting for at least 1 second. Cases where the flies were in proximity but not interacting or only fencing were not classified as aggression.

5.4.3 Quantification of behaviours

Data analysis was performed using custom Python 3.6 (`http://www.python.org/`) scripts for all experiments. For experiments in Figures 2.1 and 2.2, aggression rate was calculated as follows:

$$aggression\ rate\ =\ \frac{\#\ aggression\ frames}{\#\ copulation\ frames}$$

and the number of aggression bouts per minute as follows:

$$aggression\ bouts\ /\ min.\ =\ \frac{\#\ aggression\ bouts}{copulation\ duration\ (in\ minutes)}$$

For all other experiments, we confirmed that the amount of aggression in the first 5 minutes of copulation is sufficient to produce statistically indistinguishable results to those generated using aggression for the entirety of copulation (data not shown). Therefore, all aggression metrics were calculated and anal-

ysed within the first 5 minutes of copulation. Aggression rate was calculated as follows:

$$aggression\ rate\ =\ \frac{\#\ aggression\ frames\ in\ first\ 5\ copulation\ minutes}{18000}$$

and the number of aggression bouts per minute as follows:

$$aggression\ bouts\ /\ min.\ =\ \frac{\#\ aggression\ bouts\ in\ first\ 5\ copulation\ minutes}{5}$$

Experiments with aggression are calculated as the percentage of experiments where at least one bout of aggression occurred. Encounters are defined as moments where the non-mating female is within 4mm of the mating pair. Aggressive encounters are calculated as the percentage of encounters in which at least one aggression bout occurs. The number of aggressive bouts targeted towards males, females, or both was scored manually, and then converted to a percentage of the total amount. To get the body points along the mating female's body targeted by aggressive females, we calculated the intersection of the aggressive female's heading with the ellipsoid representation of the mating female's body. This point was then converted to an angular representation to make it female body size-agnostic, and this angle can then be mapped to the mating female's body axis. Distance walked was calculated from the start of each experiment until the end of the first copulation. We found no difference between the amount of distance walked before copulation starts and the amount of distance walked during copulation for the non-mated females (data not shown). The hatching rate is calculated as follows:

$$hatching\ rate\ =\ \frac{\#\ adults\ eclosed}{\#\ eggs\ laid}$$

For all boxplots, the outline of the box represents the interquartile range (IQR), the upper whiskers are drawn up to Q3 + (1.5 × IQR), and the lower whiskers are drawn down to Q1 − (1.5 × IQR). The line inside the box denotes the median of each sample. Each dot on the overlapping swarm plot corresponds to a fly.

5.4.4 Statistical analysis

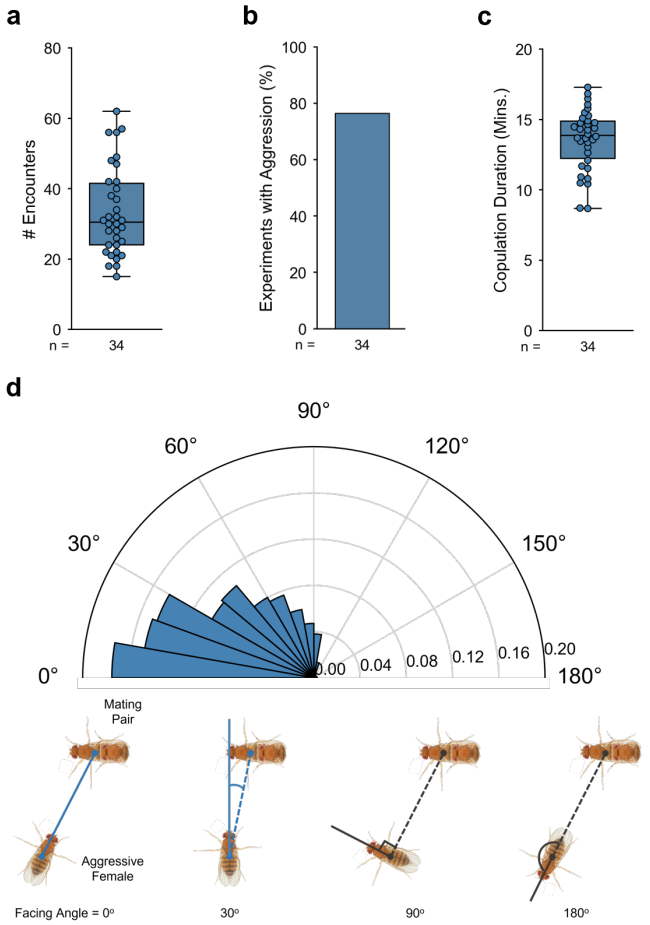
Prior to statistical testing, any outliers for aggression rate were removed from our samples. Individual data points were considered outliers if, and only if, they both lied outside the whisker range of the boxplots and their absolute z-score was equal to or higher than 3 standard deviations. Outliers never comprised more than 6% of our total samples and were excluded from any and all analyses. After discarding outliers, Levene's test was used to assess variance homogeneity, and Shapiro-Wilk and D'Agostino-Pearson tests were used to assess normality across all individual experiments. For all pairwise comparisons, if all these parametric assumptions were met, groups were compared using independent t-tests; if the groups were normally distributed but had non-homogeneous variance, comparisons were made using independent t-tests with Welch's correction for unequal variances; if none of the parametric assumptions were met, groups were compared using Mann-Whitney U tests. After testing, p-values were adjusted using Bonferroni's correction any time two or more pairwise comparisons were performed. The sample size for each comparison is indicated in each plot, and p-values, sample sizes, parametric assumptions, and statistical tests used are reported in Supplementary Tables 4.4.3 and 4.4.4.

Chapter 6

Supplementary Information

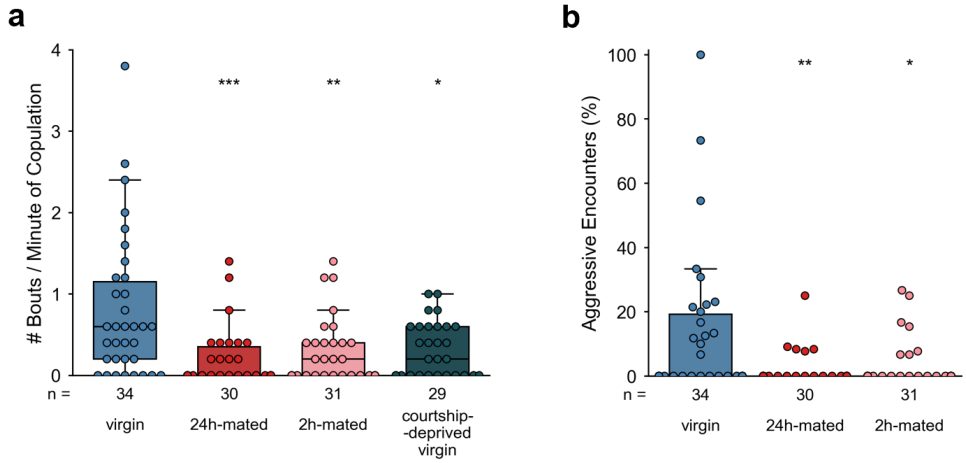
6.1 Supplementary Figures

Supplementary Figure 4.1.1



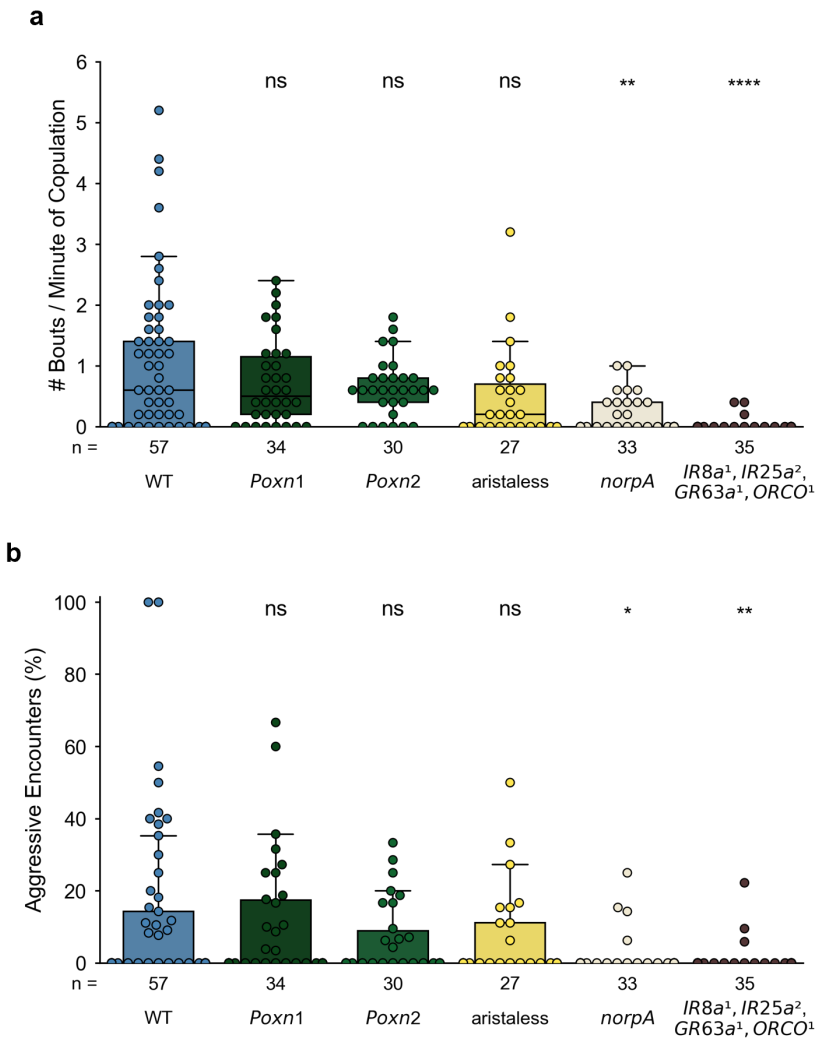
Characterization of female aggressive behaviour in the presence of a mating pair.
(a) Number of encounters occurring during copulation. Encounters are defined as moments where the female is less than 4mm away from the mating pair. **(b)** Aggression occurrence, expressed as the percentage of total experiments where aggression is observed. **(c)** Duration, in minutes, of the first copulation (target female). **(d)** Distribution of facing angles. Top: the polar axis represents the range of possible angles, while the radial axis represents the percentage of total angles that fall within any given range. Angles are binned in 10-degree intervals. Bottom: schematic of representative facing angles. Full lines represent the orientation of the aggressive female; dashed lines represent the union of both females' centroid. Blue lines match the range of angles found in the distribution.

Supplementary Figure 4.1.2



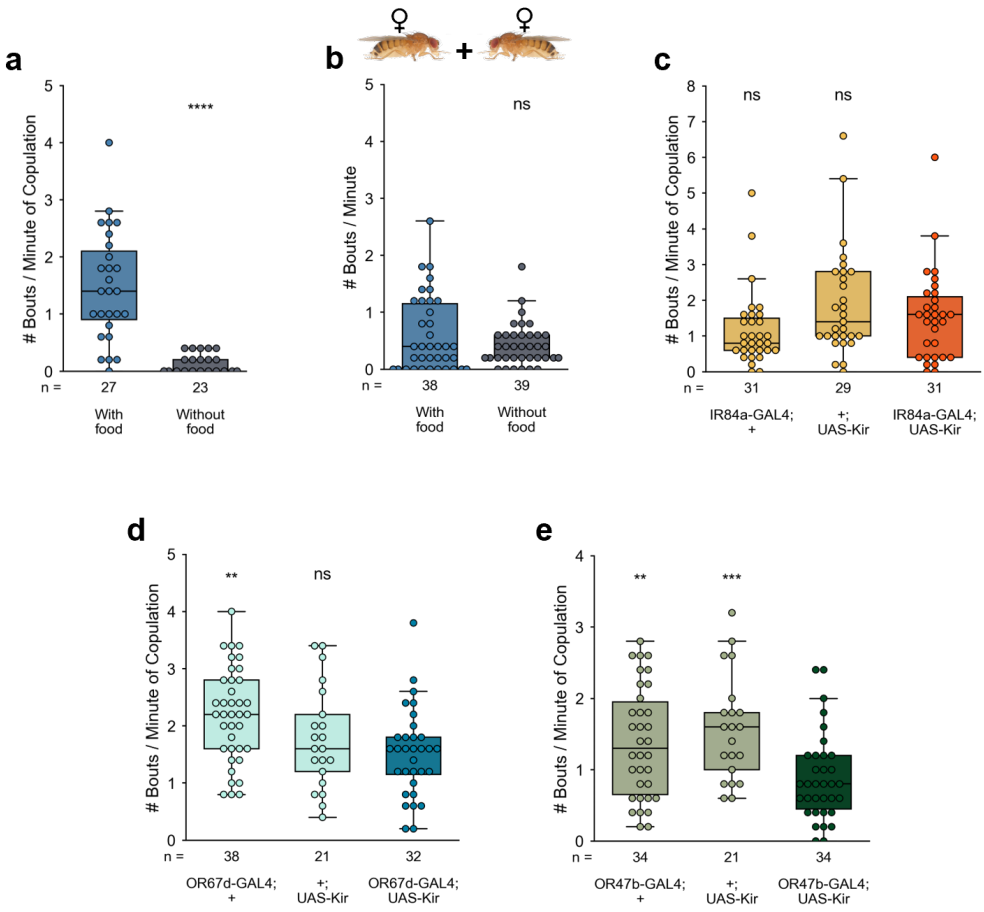
Aggression intensity is reduced by mating or lack of courtship exposure. **(a)** Number of aggression bouts per minute of copulation displayed by wild type virgin, 24h-mated, 2h-mated, and courtship-deprived virgin females towards a mating pair. **(b)** Percentage of encounters where aggression occurs. All statistical output was provided by Mann-Whitney U tests, or Student's t tests for independent groups, where applicable. To account for multiple comparisons effects, Bonferroni correction was applied post-hoc whenever two or more groups were compared; ns = not significant, *p $<$ 0.05, **p $<$ 0.01, ***p $<$ 0.001. Sample size (number of flies tested) is shown for each condition below its corresponding boxplot. See Supplementary Table 4.4.4 for exact p-values, effect sizes, and statistical tests and assumptions used.

Supplementary Figure 4.1.3



Olfaction and vision play an important role in female aggressive displays. **(a)** Number of aggression bouts per minute of copulation displayed by wild type (WT), tasteless (*Poxn1* and *Poxn2*), deaf (aristaless), blind (*norpA*), and anosmic ($IR8a^1$, $IR25a^2$, $GR63a^1$, $ORCO^1$) virgin females towards a mating pair. **(b)** Percentage of encounters where aggression occurs. All statistical output was provided by Mann-Whitney U tests, or Student's t tests for independent groups, where applicable. To account for multiple comparisons effects, Bonferroni correction was applied post-hoc whenever two or more groups were compared; ns = not significant, *p < 0.05, **p < 0.01, ****p < 0.0001. Sample size (number of flies tested) is shown for each condition below its corresponding boxplot. See Supplementary Table 4.4.2 for detailed fly genotypes, and Supplementary Table 4.4.4 for exact p-values, effect sizes, and statistical tests and assumptions used.

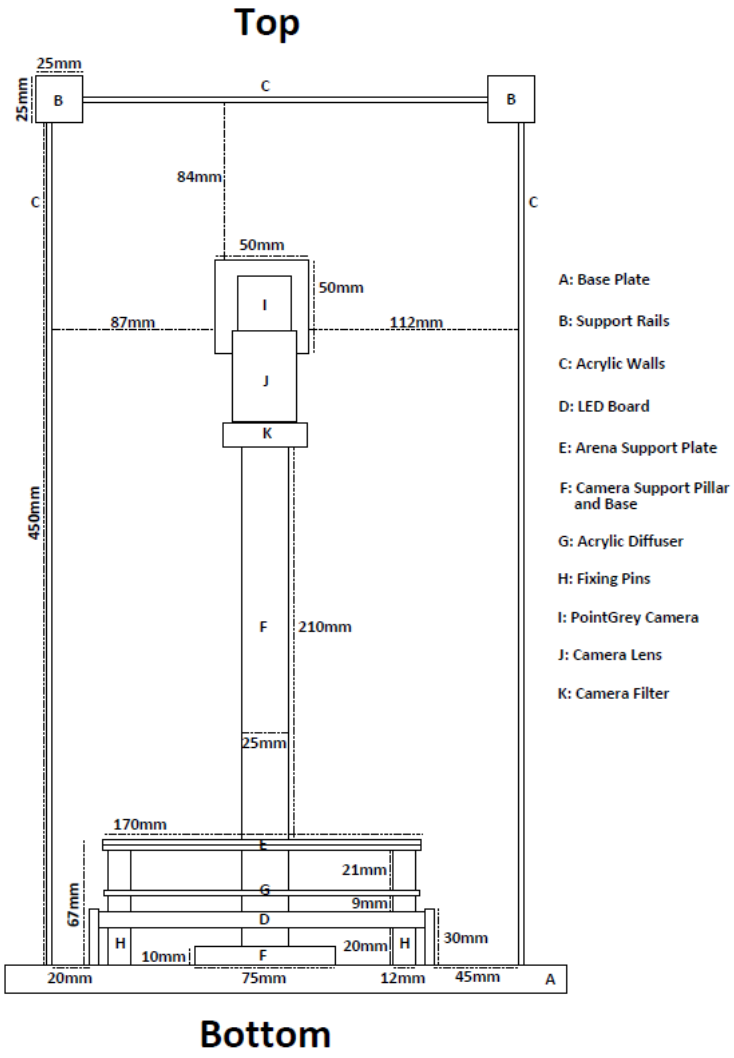
Supplementary Figure 4.1.4



Presence of food odour and OR47b activity play an important role in female aggressive displays.
Number of aggression bouts per minute in the following conditions: **(a)** Wild type flies in the absence or presence of food odour. **(b)** Wild type female pairs, without a male, in the absence or presence of food odour. **(c)** Silenced IR84a flies and respective controls in the presence of food odour. **(d)** Silenced OR67d flies and respective controls in the presence of food odour. **(E)** Silenced OR47b flies and respective controls in the presence of food odour. All statistical output was provided by Mann-Whitney U tests, or Student's t tests for independent groups, where applicable. To account for multiple comparisons effects, Bonferroni correction was applied post-hoc whenever two or more groups were compared; ns = not significant, **p $<$ 0.01, ***p $<$ 0.001, ****p $<$ 0.0001. Sample size (number of flies tested) is shown for each condition below its corresponding boxplot. See Supplementary Table 4.4.2 for detailed fly genotypes, and Supplementary Table 4.4.4 for exact p-values, effect sizes, and statistical tests and assumptions used.

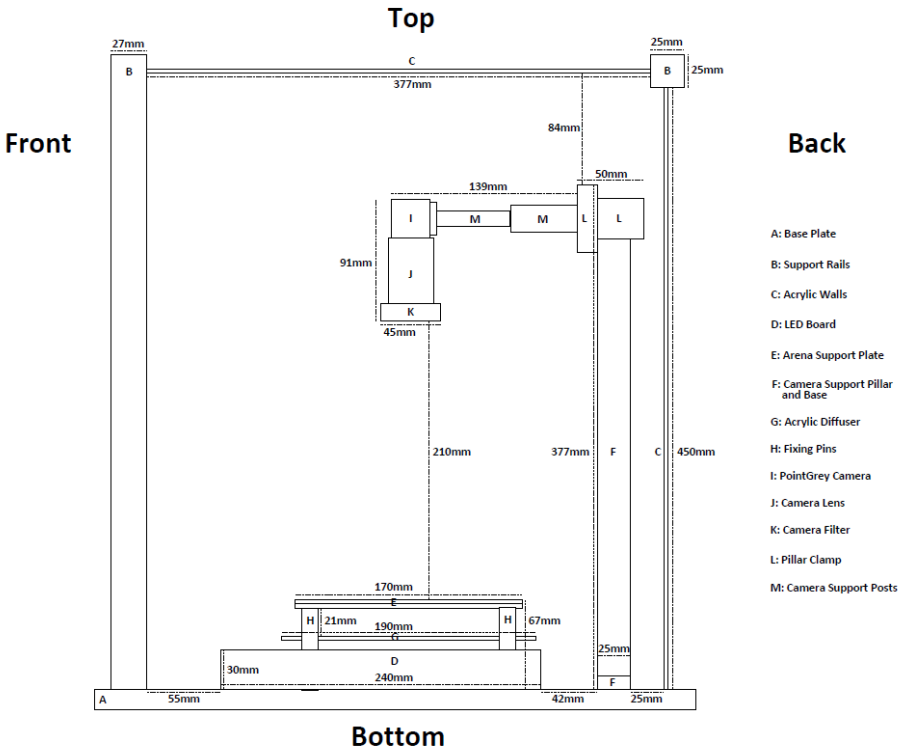
6.2 Supplementary Diagrams

Supplementary Diagram 4.2.1



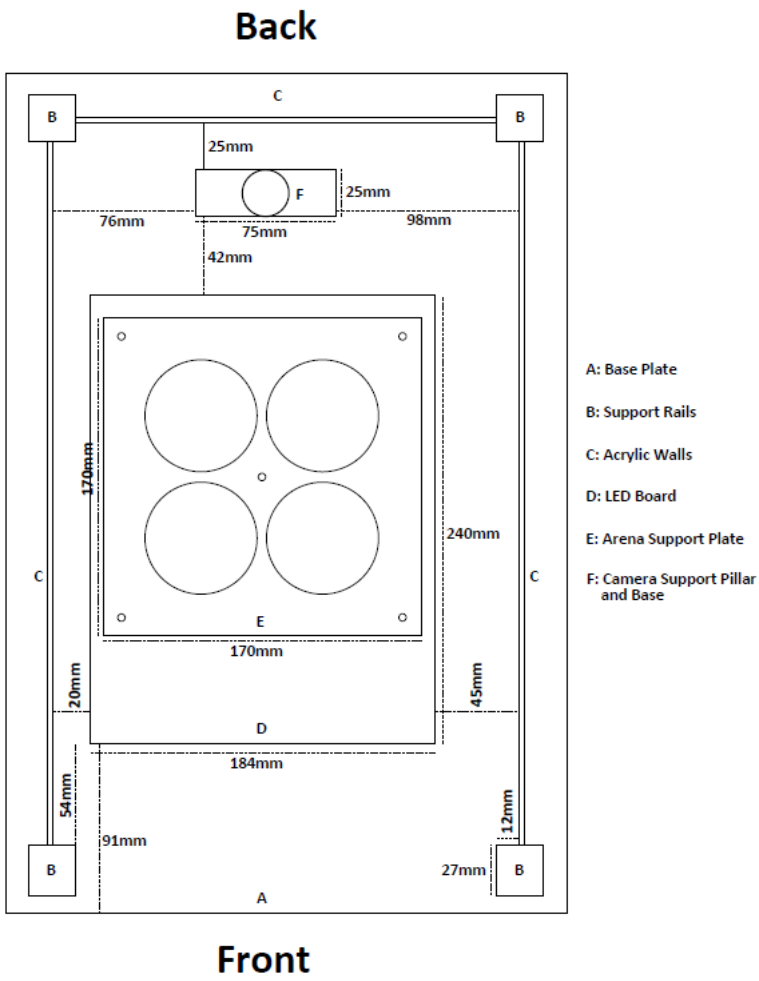
Schematic representation of the behavioural setup used in all experiments. Front view. To see a list of parts used in this construction, please refer to Supplementary Table 4.4.5. Drawn in Inkscape software at 1:2 scale. All measurements are given in millimeters (mm).

Supplementary Diagram 4.2.1 (*continued*)



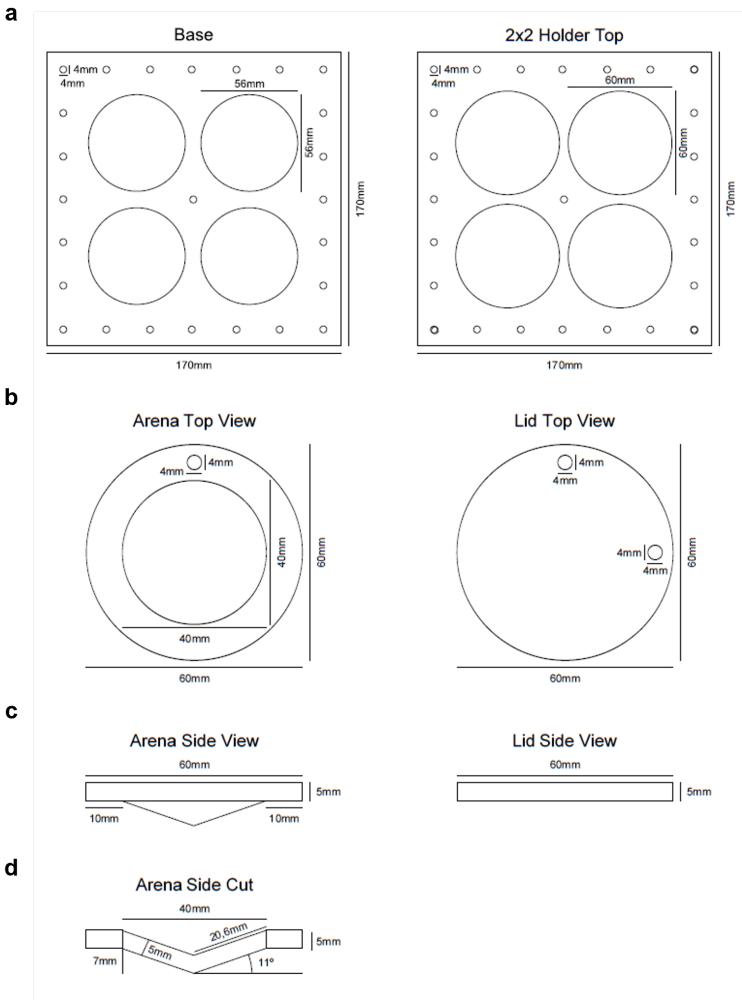
Schematic representation of the behavioural setup used in all experiments. Lateral view. To see a list of parts used in this construction, please refer to Supplementary Table 4.4.5. Drawn in Inkscape software at 1:2 scale. All measurements are given in millimeters (mm).

Supplementary Diagram 4.2.1 (*continued*)



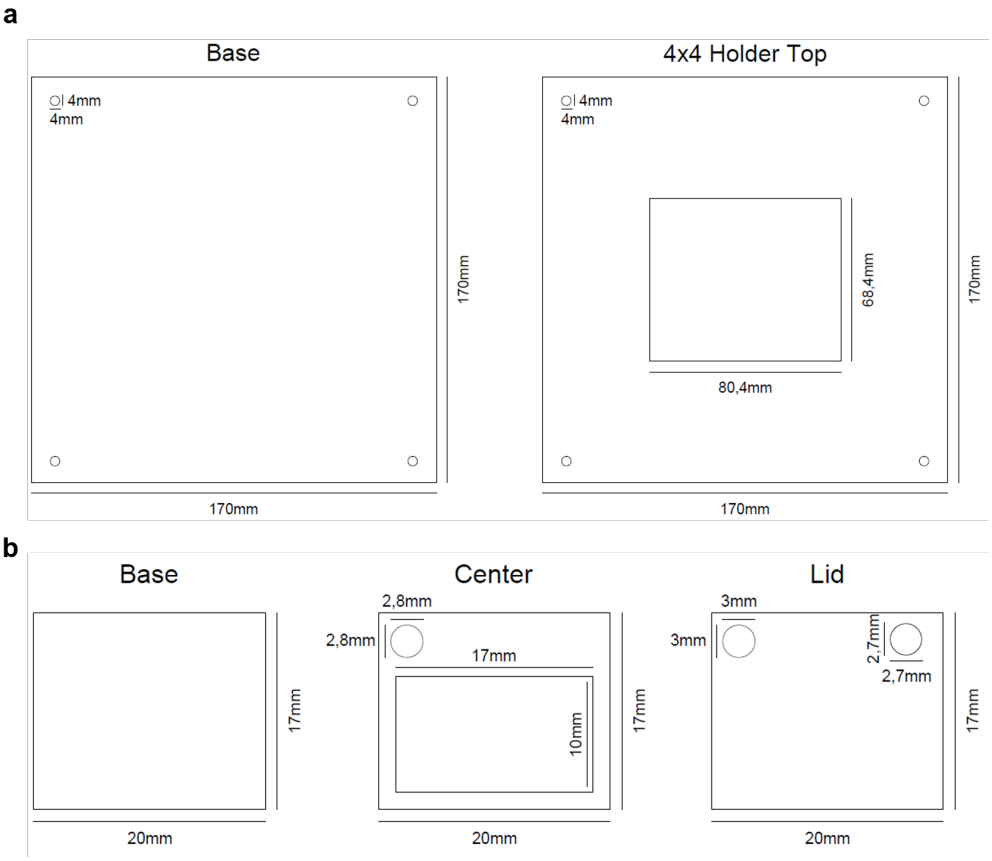
Schematic representation of the behavioural setup used in all experiments. Top view. To see a list of parts used in this construction, please refer to Supplementary Table 4.4.5. Drawn in Inkscape software at 1:2 scale. All measurements are given in millimeters (mm).

Supplementary Diagram 4.2.2



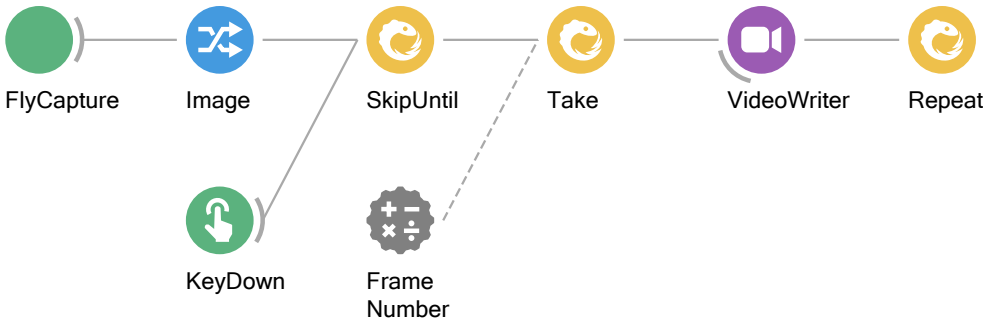
Schematic representation of the behavioural arenas used.
Related to experiments from Figures 2.1, 2.2, 3.1, 3.2, as well as Supplementary Figures 4.1.1, 4.1.2, and 4.1.3. **(a)** Acrylic bases serving as arena supports, allowing simultaneous recording of 4 arenas. **(b)** Top view of the behavioural arena (left) and lid (right). **(c)** Lateral view of the behavioural arena (left) and lid (right). **(d)** Transversal cut of the behavioural arena. A Thorlabs M4×0.7×12mm setscrew is placed in the top orifice of the lid, providing an anchor point for holding the lid in place and allowing it to easily slide open or closed. The right-most orifice in on the lid allows for aspiration of flies into the arena. Both bases were laser-cut from 3mm-thick white opaque acrylic. Arenas are made of mechanically-bored white polyoxymethylene, while the lids are made of laser-cut 5mm-thick clear acrylic. For camera settings used in movie acquisition of experiments using these arenas, please refer to Supplementary Table 4.4.6. Drawn in Inkscape software at 1:2 scale ((**a**)), or 1:1 scale ((**b**), **(c)**, and **(d)**). All measurements are given in millimeters (mm).

Supplementary Diagram 4.2.3



Schematic representation of the behavioural arenas used.
Related to experiments from Figures 2.3 and 3.3, as well as Supplementary Figure 4.1.4. **(a)** Acrylic bases serving as arena supports, allowing simultaneous recording of 16 arenas. **(b)** Top view of each of the acrylic parts that comprise the behavioural arena. Top and middle sections are glued together to form a chamber, which is then covered by the movable lid. A Thorlabs M3×0.5×6mm stainless steel setscrew is placed in the top left orifice of the bottom and middle sections, providing an anchor point for holding the lid in place and allowing it to easily slide open or closed. The right-most orifice in on the lid allows for aspiration of flies into the arena. Both bases were laser-cut from 3mm-thick white opaque acrylic. All parts of the arenas were laser-cut from 3mm-thick clear acrylic. For camera settings used in movie acquisition of experiments using these arenas, please refer to Supplementary Table 4.4.6. Drawn in Inkscape software at 1:2 scale (**(a)**), or 2:1 scale (**(b)**). All measurements are given in millimeters (mm).

Supplementary Diagram 4.2.4



Bonsai workflow used for video acquisition.
Each coloured circle represents a Bonsai operator that contributes to the workflow. "FlyCapture" and "Image" operators receive input from the camera; "KeyDown" and "SkipUntil" prevent input frames from being recorded until a user-defined keyboard key is pressed, giving full control of when to start recording; "Frame Number" defines the amount of time (in frames) to record for (e.g., 108000 frames for a 30-minute video); "Take" ensures that the recording automatically stops after the set amount of frames have been recorded, without requiring additional user input; "VideoWriter" saves all incoming input frames to a user-determined local folder; "Repeat" allows for the workflow to be immediately reusable after each recording ends. The "FrameRate" and "FrameSize" attributes (not shown) of the "VideoWriter" operator must match the camera settings (see Supplementary Table 4.4.6).

6.3 Supplementary Videos

Videos can be accessed trough this link: `https://www.biorxiv.org/content/10.1101/2022.02.07.479369v1.supplementary-material`

Supplementary Video 4.3.1

Example instance of a non-mating female displaying three consecutive bouts of aggressive behaviour towards the mating pair. Related to Figures 2.1, 2.2, 3.1, and 3.2, as well as Supplementary Figures 4.1.1, 4.1.2, and 4.1.3. Each bout is identified by a co-occurring red circle labelled "Aggression" around the flies.

Supplementary Video 4.3.2

Example instance of a non-mating female displaying three consecutive bouts of aggressive behaviour towards the mating pair in a new, more spatially constrained arena. Related to Figures 2.3 and 3.3, as well as Supplementary Figure 4.1.4. Each bout is identified by a co-occurring red circle labelled "Aggression" around the flies.

Supplementary Video 4.3.3

Example instance of a non-mating female separated from the mating pair by a nylon mesh. Related to Figures 2.3 and 3.3, as well as Supplementary Figure 4.1.4. The mesh is present since the start of the experiment, when flies are introduced to the arena, and removed after 30 minutes, thus allowing for courtship and mating of the second, isolated female.

6.4 Supplementary Tables

Supplementary Table 4.4.1. Fly stocks.

Stocks	Source	Reference
Wild type (DL strain)	-	Dickinson, 1999
w^{1118} ; PoxnΔ$^{[M22-B5]}$; Δ$^{[SfoBs105]}$	-	Boll and Noll, 2002
w^{1118} ; PoxnΔ$^{[M22-B5]}$; Δ$^{[SfoBs127]}$	-	Boll and Noll, 2002
w[*], norpA^{36} ; + ; +	BDSC_9048	Pearn et al., 1996
IR8a^{1}; IR25a^{2}; Orco^{1}, GR63a^{1}	-	Ramdya et al., 2015
w[*] ; UAS-Kir2.1.EGFP ; +	BDSC_6596	Baines et al., 2001
w[*] ; IR84a-GAL4 ; TM2/TM6b, Tb^{1}	BDSC_41734	Silbering et al., 2011
w[*], OR67d-GAL4 ; + ; +	BDSC_9997	Fishilevich and Vosshall, 2005
w[*] ; OR47b-GAL4; +	BDSC_9983	Vosshall et al., 2000

Note: BDSC stands for Bloomington Drosophila Stock Centre.

Supplementary Table 4.4.2. Full genotypes of flies used.

Figures	Genotypes
Figure 1	Wild type (DL strain)
Figure 2	Wild type (DL strain)
Figure 3	Wild type (DL strain)
Figure 4	Control: wild type (DL strain) Poxn1 mutant: w^{1118} ; $PoxnΔ^{[M22\text{-}B5]}$; $Δ^{[SfoBs105]}$ / $Δ^{[SfoBs127]}$ Poxn2 mutant: w^{1118} ; $PoxnΔ^{[M22\text{-}B5]}$; $Δ^{[SfoBs127]}$ / $Δ^{[SfoBs105]}$ Aristaless: wild type (DL strain) with both aristae removed Blind mutant: *w[*], norpA*^{36} ; + ; + Anosmic mutant: *IR8a*^{1}*; IR25a*^{2}*; Orco*^{1}*, GR63a*^{1}
Figure 5a-b	Wild type (DL strain)
Figure 5c	Control 1: *w[*] ; IR84a-GAL4 /* + ; + Control 2: *w[*] ;* + */ UAS-Kir2.1-EGFP ;* + Test: *w[*] ; IR84a-GAL4 / UAS-Kir2.1-EGFP ;* +
Figure 5d	Control 1: *w[*], OR67d-GAL4 /* + ; + ; + Control 2: *w[*] ;* + */ UAS-Kir2.1-EGFP ;* + Test: *w[*], OR67d-GAL4 /* + ; + */ UAS-Kir2.1-EGFP ;* +
Figure 5e	Control 1: *w[*] ; OR47b-GAL4 /* + ; + Control 2: *w[*] ;* + */ UAS-Kir2.1-EGFP ;* + Test: *w[*] ; OR47b-GAL4 / UAS-Kir2.1-EGFP ;* +
Supplementary Figure 1	Wild type (DL strain)
Supplementary Figure 2	Wild type (DL strain)
Supplementary Figure 3	Control: wild type (DL strain) Poxn1 mutant: w^{1118} ; $PoxnΔ^{[M22\text{-}B5]}$; $Δ^{[SfoBs105]}$ / $Δ^{[SfoBs127]}$ Poxn2 mutant: w^{1118} ; $PoxnΔ^{[M22\text{-}B5]}$; $Δ^{[SfoBs127]}$ / $Δ^{[SfoBs105]}$ Aristaless: wild type (DL strain) with both aristae removed Blind mutant: *w[*], norpA*^{36} ; + ; + Anosmic mutant: *IR8a*^{1}*; IR25a*^{2}*; Orco*^{1}*, GR63a*^{1}
Supplementary Figure 4a-b	Wild type (DL strain)
Supplementary Figure 4c	Control 1: *w[*] ; IR84a-GAL4 /* + ; + Control 2: *w[*] ;* + */ UAS-Kir2.1-EGFP ;* + Test: *w[*] ; IR84a-GAL4 / UAS-Kir2.1-EGFP ;* +
Supplementary Figure 4d	Control 1: *w[*], OR67d-GAL4 /* + ; + ; + Control 2: *w[*] ;* + */ UAS-Kir2.1-EGFP ;* + Test: *w[*], OR67d-GAL4 /* + ; + */ UAS-Kir2.1-EGFP ;* +
Supplementary Figure 4e	Control 1: *w[*] ; OR47b-GAL4 /* + ; + Control 2: *w[*] ;* + */ UAS-Kir2.1-EGFP ;* + Test: *w[*] ; OR47b-GAL4 / UAS-Kir2.1-EGFP ;* +

Supplementary Table 4.4.3. Statistical details for main figures.

Figure	Groups	Sample size	Normally distributed[a]	Equal variance[b]	Statistical test	p-value	Effect size[c]
2a	a) wild type female with aggression b) wild type female without aggression (partition)	34 28	no no	no	Mann-Whitney test	0.566732 (ns)	0.026
	a) wild type female with aggression c) wild type female without aggression (couple)	34 40	no no	yes	Mann-Whitney test	0.033061 (*)	0.176
2b	a) wild type female with aggression b) wild type female without aggression (partition)	34 28	no no	yes	Mann-Whitney test	0.139606 (ns)	- 0.107
2c	a) wild type female with aggression b) wild type female without aggression (partition)	34 28	yes yes	yes	Two sample t-test	0.178776 (ns)	- 0.319
	a) wild type female with aggression c) wild type female without aggression (couple)	34 40	yes no	yes	Mann-Whitney test	0.591032 (ns)	- 0.064
2d	a) wild type female with aggression b) wild type female without aggression (partition)	34 28	yes yes	yes	Two sample t-test	1.0 (ns)	0.130
	a) wild type female with aggression c) wild type female without aggression (couple)	34 40	yes no	yes	Mann-Whitney test	0.428794 (ns)	0.034
2e	a) wild type female with aggression b) wild type female without aggression (partition)	34 28	no yes	yes	Mann-Whitney test	0.391206 (ns)	0.146
2f	a) wild type female with aggression b) wild type female without aggression (partition)	34 28	yes yes	yes	Two sample t-test	0.749854 (ns)	- 0.021
3a	a) wild type virgin female b) wild type 24h-mated female	34 30	no no	no	Mann-Whitney test	0.000621 (***)	- 1.0
	a) wild type virgin female c) wild type 2h-mated female	34 31	no no	no	Mann-Whitney test	0.001358 (**)	- 0.950
	a) wild type virgin female d) wild type added virgin female	34 29	no no	no	Mann-Whitney test	0.003371 (**)	- 0.888
3b	a) wild type virgin female b) wild type 24h-mated female	34 30	yes no	yes	Mann-Whitney test	0.329374 (ns)	0.134
	a) wild type virgin female c) wild type 2h-mated female	34 31	yes no	yes	Mann-Whitney test	0.879943 (ns)	- 0.071
4a	a) wild type virgin female b) tasteless mutant 1 virgin female	57 34	no no	yes	Mann-Whitney test	1.0 (ns)	- 0.118
	a) wild type virgin female b) tasteless mutant 2 virgin female	57 30	no no	yes	Mann-Whitney test	1.0 (ns)	0.335
	a) wild type virgin female b) deaf virgin female	57 27	no no	no	Mann-Whitney test	0.178375 (ns)	- 0.757
	a) wild type virgin female b) blind mutant virgin female	57 33	no no	no	Mann-Whitney test	0.005105 (**)	- 1.0
	a) wild type virgin female b) anosmic mutant virgin female	57 35	no no	no	Mann-Whitney test	1.869×10^{-7} (****)	- 1.0
4b	a) wild type virgin female b) tasteless mutant 1 virgin female	57 34	yes yes	yes	Two sample t-test	1.0 (ns)	- 0.076
	a) wild type virgin female b) tasteless mutant 2 virgin female	57 30	no no	yes	Mann-Whitney test	1.0 (ns)	- 0.054
	a) wild type virgin female b) deaf virgin female	57 27	yes yes	yes	Two sample t-test	1.0 (ns)	- 0.148
	a) wild type virgin female b) blind mutant virgin female	57 33	yes yes	yes	Two sample t-test	1.0 (ns)	- 0.186
	a) wild type virgin female b) anosmic mutant virgin female	57 35	yes yes	yes	Two sample t-test	1.0 (ns)	- 0.009
5a	a) wild type virgin female (with food) b) wild type virgin female (without food)	27 23	no no	no	Mann-Whitney test	2.941×10^{-8} (****)	- 1.0
5b	a) wild type virgin female (with food) b) wild type virgin female (without food)	38 39	no no	no	Mann-Whitney test	0.247603 (ns)	0.886
5c	a) IR84a > Kir2.1 silencing b) Gal4 control	31 31	no no	yes	Mann-Whitney test	0.159089 (ns)	0.859
	a) IR84a > Kir2.1 silencing b) UAS control	31 29	no no	yes	Mann-Whitney test	0.700511 (ns)	- 0.207
5d	a) OR67d > Kir2.1 silencing b) Gal4 control	32 38	no yes	yes	Mann-Whitney test	0.074072 (ns)	- 0.395
	a) OR67d > Kir2.1 silencing b) UAS control	32 21	no no	yes	Mann-Whitney test	0.591601 (ns)	- 0.241
5e	a) OR47b > Kir2.1 silencing b) Gal4 control	34 34	no no	yes	Mann-Whitney test	0.037607 (*)	- 0.415
	a) OR47b > Kir2.1 silencing b) UAS control	34 21	no no	yes	Mann-Whitney test	0.014572 (*)	- 0.398

Supplementary Table 4.4.4. Statistical details for supplementary figures.

Figure	Groups	Sample size	Normally distributed[a]	Equal variance[b]	Statistical test	p-value	Effect size[c]
S2a	a) wild type virgin female b) wild type 24h-mated female	34 30	no no	no	Mann-Whitney test	0.000663 (***)	- 1.0
	a) wild type virgin female c) wild type 2h-mated female	34 31	no no	no	Mann-Whitney test	0.009462 (**)	- 0.667
	a) wild type virgin female d) wild type added virgin female	34 29	no no	no	Mann-Whitney test	0.049136 (*)	- 0.667
S2b	a) wild type virgin female b) wild type 24h-mated female	34 30	no no	no	Mann-Whitney test	0.007610 (**)	NA
	a) wild type virgin female c) wild type 2h-mated female	34 31	no no	no	Mann-Whitney test	0.038226 (*)	NA
S3a	a) wild type virgin female b) tasteless mutant 1 virgin female	57 34	no no	yes	Mann-Whitney test	1.0 (ns)	- 0.167
	a) wild type virgin female b) tasteless mutant 2 virgin female	57 30	no no	no	Mann-Whitney test	1.0 (ns)	0.0
	a) wild type virgin female b) deaf virgin female	57 27	no no	yes	Mann-Whitney test	0.236137 (ns)	- 0.667
	a) wild type virgin female b) blind mutant virgin female	57 33	no no	no	Mann-Whitney test	0.001278 (***)	- 1.0
	a) wild type virgin female b) anosmic mutant virgin female	57 35	no no	no	Mann-Whitney test	2.247×10^{-7} (****)	- 1.0
S3b	a) wild type virgin female b) tasteless mutant 1 virgin female	57 34	no no	yes	Mann-Whitney test	1.0 (ns)	NA
	a) wild type virgin female b) tasteless mutant 2 virgin female	57 30	no no	yes	Mann-Whitney test	1.0 (ns)	NA
	a) wild type virgin female b) deaf virgin female	57 27	no no	yes	Mann-Whitney test	1.0 (ns)	NA
	a) wild type virgin female b) blind mutant virgin female	57 33	no no	no	Mann-Whitney test	0.020358 (*)	NA
	a) wild type virgin female b) anosmic mutant virgin female	57 35	no no	no	Mann-Whitney test	0.004451 (**)	NA
S4a	a) wild type virgin female (with food) b) wild type virgin female (without food)	27 23	yes no	no	Mann-Whitney test	4.357×10^{-9} (****)	- 1.0
S4b	a) wild type virgin female (with food) b) wild type virgin female (without food)	38 39	no no	yes	Mann-Whitney test	0.419892 (ns)	0.0
S4c	a) IR84a > Kir2.1 silencing b) Gal4 control	31 31	no no	yes	Mann-Whitney test	0.172588 (ns)	1.0
	a) IR84a > Kir2.1 silencing b) UAS control	31 29	no no	yes	Mann-Whitney test	0.410530 (ns)	0.143
S4d	a) OR67d > Kir2.1 silencing b) Gal4 control	32 38	yes yes	yes	Two sample t-test	0.001858 (**)	- 0.273
	a) OR67d > Kir2.1 silencing b) UAS control	32 21	yes yes	yes	Two sample t-test	0.544273 (ns)	0.0
S4e	a) OR47b > Kir2.1 silencing b) Gal4 control	34 34	no yes	no	Mann-Whitney test	0.008534 (**)	- 0.385
	a) OR47b > Kir2.1 silencing b) UAS control	34 21	no yes	yes	Mann-Whitney test	0.000599 (***)	- 0.5

ns = not significant
* = p-value < 0.05
** = p-value < 0.01
*** = p-value < 0.001
**** = p-value < 0.0001
NA = Not Applicable
[a] To test for normal distribution: Shapiro's test and D'Agostino's test.
[b] To test for the homogeneity of variance: Levene´s test for non-normally distributed samples and Bartlett´s test for normally distributed samples.
[c] To calculate effect size:

$$median\ fold\ change\ =\ \frac{median_{test}\ -\ median_{control}}{median_{control}}$$

Supplementary Table 4.4.5. Materials and parts used in building the behavioural setup.

Company	Quantity	Description	Reference
Computar	1	H0514-MP2 (1/2" 5mm f1.4 w/locking Iris & Focus, Megapixel (C Mount))	http://computar.com/product/551/H0514-MP2
Edmund Optics	1	Flea®3 FL3-U3-32S2M-CS 1/2.8" Monochrome USB 3.0 Camera	https://www.edmundoptics.eu/cameras/usb-cameras/point-grey-flea3-usb-3-0-cameras/86765
Edmund Optics	1	UV/VIS Cut-Off M43.0 x 0.75 Imaging Filter	https://www.edmundoptics.eu/optics/optical-filters/imaging-filters/imaging-filters/89839
Thorlabs	1	AP6M4M - Adapter with External M6 x 1.0 Threads and External M4 x 0.7 Threads	https://www.thorlabs.com/thorproduct.cfm?partnumber=AP6M4M
Thorlabs	1	BA1 - Mounting Base, 1" x 3" x 3/8"	https://www.thorlabs.com/thorproduct.cfm?partnumber=BA1
Thorlabs	1	C1001/M - Post Mounting Clamp for Ø25 mm Post, Metric	https://www.thorlabs.com/thorproduct.cfm?partnumber=C1001/M
Thorlabs	1	MB3045/M - Aluminum Breadboard, 300 mm x 450 mm x 12.7 mm, M6 Taps	https://www.thorlabs.com/thorproduct.cfm?partnumber=MB3045/M
Thorlabs	1	PH40/M-P5 - Ø12.7 mm Post Holders, Spring-Loaded Hex-Locking Thumbscrew, L=40 mm, 5 Pack	https://www.thorlabs.com/thorproduct.cfm?partnumber=PH40/M-P5
Thorlabs	4	RM1G - 1" Construction Cube, Three 1/4" (M6) Counterbored Holes	https://www.thorlabs.com/thorproduct.cfm?partnumber=RM1G
Thorlabs	2	RS100/M - Ø25.0 mm Pillar Post, M6 Taps, L = 100 mm, M4 Adapter Included	https://www.thorlabs.com/thorproduct.cfm?partnumber=RS100/M
Thorlabs	1	RS150/M - Ø25.0 mm Pillar Post, M6 Taps, L = 150 mm, M4 Adapter Included	https://www.thorlabs.com/thorproduct.cfm?partnumber=RS150/M
Thorlabs	1	SS3M6 - M3 x 0.5 Stainless Steel Setscrew, 6 mm Long, 50 Pack	https://www.thorlabs.com/thorproduct.cfm?partnumber=SS3M6
Thorlabs	1	SS4MS12 - M4 x 0.7 Stainless Steel Setscrew, 12 mm Long, 50 Pack	https://www.thorlabs.com/thorproduct.cfm?partnumber=SS4MS12
Thorlabs	1	SS6MS20 - M6 x 1.0 Stainless Steel Setscrew, 20 mm Long, Pack of 25	https://www.thorlabs.com/thorproduct.cfm?partnumber=SS6MS20
Thorlabs	3	TR20/M - Ø12.7 mm Optical Post, SS, M4 Setscrew, M6 Tap, L = 20 mm	https://www.thorlabs.com/thorproduct.cfm?partnumber=TR20/M
Thorlabs	1	TR40/M-P5 - Ø12.7 mm Optical Post, SS, M4 Setscrew, M6 Tap, L = 40 mm, 5 Pack	https://www.thorlabs.com/thorproduct.cfm?partnumber=TR40/M-P5
Thorlabs	1	TR75/M-P5 - Ø12.7 mm Optical Post, SS, M4 Setscrew, M6 Tap, L = 75 mm, 5 Pack	https://www.thorlabs.com/thorproduct.cfm?partnumber=TR75/M-P5
Thorlabs	1	TR100/M-P5 - Ø12.7 mm Optical Post, SS, M4 Setscrew, M6 Tap, L = 100 mm, 5 Pack	https://www.thorlabs.com/thorproduct.cfm?partnumber=TR100/M-P5
Thorlabs	1	TR150/M-P5 - Ø12.7 mm Optical Post, SS, M4 Setscrew, M6 Tap, L = 150 mm, 5 Pack	https://www.thorlabs.com/thorproduct.cfm?partnumber=TR150/M-P5
Thorlabs	2	XE25L225/M - 25 mm Construction Rail, L = 225 mm	https://www.thorlabs.com/thorproduct.cfm?partnumber=XE25L225/M
Thorlabs	2	XE25L375/M - 25 mm Construction Rail, L = 375 mm	https://www.thorlabs.com/thorproduct.cfm?partnumber=XE25L375/M
Thorlabs	4	XE25L450/M - 25 mm Construction Rail, L = 450 mm	https://www.thorlabs.com/thorproduct.cfm?partnumber=XE25L450/M

Supplementary Table 4.4.6. Camera settings for video acquisition.

Software	Parameter	Value
PointGrey FlyCapture2	Brightness	0,000
PointGrey FlyCapture2	Exposure	1,322
PointGrey FlyCapture2	Sharpness	1220
PointGrey FlyCapture2	Gamma	1,034
PointGrey FlyCapture2	Shutter	15,648
PointGrey FlyCapture2	Gain	10,200
PointGrey FlyCapture2	FrameRate	60
PointGrey FlyCapture2	Image Left	580
PointGrey FlyCapture2	Image Top	364
PointGrey FlyCapture2	Image Width	960
PointGrey FlyCapture2	Image Height	940
Harp LED Interface	LED Power	120

Software	Parameter	Value
PointGrey FlyCapture2	Brightness	0,000
PointGrey FlyCapture2	Exposure	1,322
PointGrey FlyCapture2	Sharpness	1220
PointGrey FlyCapture2	Gamma	1,301
PointGrey FlyCapture2	Shutter	15,648
PointGrey FlyCapture2	Gain	10,200
PointGrey FlyCapture2	FrameRate	60
PointGrey FlyCapture2	Image Left	448
PointGrey FlyCapture2	Image Top	230
PointGrey FlyCapture2	Image Width	1248
PointGrey FlyCapture2	Image Height	1010
Harp LED Array	LED Power	120

Camera settings used to record behaviour. Left table: settings for experiments related to Figures 2.1, 2.2, 3.1, and 3.2, as well as Supplementary Figures 4.1.1, 4.1.2, and 4.1.3; right table: settings for experiments related to Figures 2.3 and 3.3, as well as Supplementary Figure 4.1.4.

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